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REVIEW

Comparative Aspects of Animal Oogenesis

Nina Matova*,[1] and Lynn Cooley†,[2]

**Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06511; and †Departments of Genetics and Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520*

INTRODUCTION

All metazoans that reproduce sexually have the ability to form gametes. Both types of gametes, the egg and the sperm, arise from germ cells, undergo a unique program of differentiation, and are destined to unite. The outcome of their union, the zygote, is a cell possessing the tremendous potential to build a new individual that expresses, maintains, and propagates characteristics of the species.The focus of this review is the development of the egg in animals, beginning with the formation of primordial germ cells in embryos. This process, known as oogenesis, has long captivated the attention of developmental biologists who have come to realize that the zygote inherits from the egg not only genetic material but also its cytoplasm. This maternal cytoplasm supports to varying degrees the development of the early embryo and is the basis for the conclusion that embryogenesis actually begins during oogenesis. A wealth of experimental evidence shows that mechanisms for establishing the germline and carrying out oogenesis in evolutionarily distant animals follow certain common themes. Several conserved principles and the known molecular mechanisms behind them are discussed below.

THE GERMLINE IS FOUNDED BY PRIMORDIAL GERM CELLS

Gametes develop from primordial germ cells (PGCs) that are set aside early in embryogenesis. The origin of primordial germ cells has been a question of central importance in developmental biology. The answer was found in a defined area of the egg, the germ plasm, in many animals including three widely used experimental organisms: the hermaphrodite nematode *Caenorhabditis elegans* (*C. elegans*), the fruit fly *Drosophila melanogaster*, and the frog *Xenopus laevis* (reviewed in Ikenishi, 1998). The cells that inherit this localized cytoplasm during the very first embryonic divisions give rise to PGCs. Germ plasm can be identified morphologically by the presence of conspicuous membrane-unbound organelles with an electron-dense granulofibrillar appearance called germ granules. They are also known as P granules in *C. elegans*, polar granules in *Drosophila*, and germinal granules in *Xenopus* (Mahowald, 1962; Czolowska, 1969; Mahowald and Hennen, 1971; Williams and Smith, 1971; Wolf *et al*., 1983). Germ plasm contains large aggregations of mitochondria that are intermingled within germ granules. The identification of mutants with absent or defective germ granules (Williamson and Lehmann, 1996; Kawasaki *et al*., 1998) and formation of PGCs at ectopic sites following germ-plasm transplantation (Illmensee and Mahowald, 1974) have supported the belief that these organelles carry the germline "determinants."

P granules are present in the *C. elegans* zygote, P0 (Fig. 1A) (Strome and Wood, 1983; Wolf *et al*., 1983). They are asymmetrically partitioned to the germ-cell lineage starting with the first embryonic division and are finally delivered to the primordial germ cell, P4. P4 divides only once during embryogenesis, while the P4 daughters, Z2 and Z3 (also called PGCs), divide during larval stages to produce about 1500 germ cells in the adult hermaphrodite. P granules are found in all descendants of P4 with the exception of the mature sperm.

In *Drosophila*, the germ plasm is localized at the posterior of the egg (Fig. 1B). The initial divisions of the zygote are solely nuclear; they occur in the absence of cytokinesis and the embryo develops as a syncytium. The first 10 nuclei that migrate to the posterior end of the embryo are surrounded by cell membranes to form pole cells. This precocious cellularization segregates the polar granules into the pole cells, away from the somatic cells that form subsequently. Pole cells divide asynchronously to produce about 40 cells (Sonnenblick, 1950), a fraction of which later become the primordial germ cells (Kobayashi *et al*., 1993). The polar granules are transformed during gastrulation into nuage, an electron-dense fibrous material often associated with mitochondria and localized perinuclearly (Mahowald,

[1] Present address: Molecular Biology Program, Sloan-Kettering Institute, 1275 York Ave., New York, NY 10021. E-mail: matovan@mskcc.org.
[2] To whom correspondence should be addressed. Fax: (203) 785-6333. E-mail: lynn.cooley@yale.edu.

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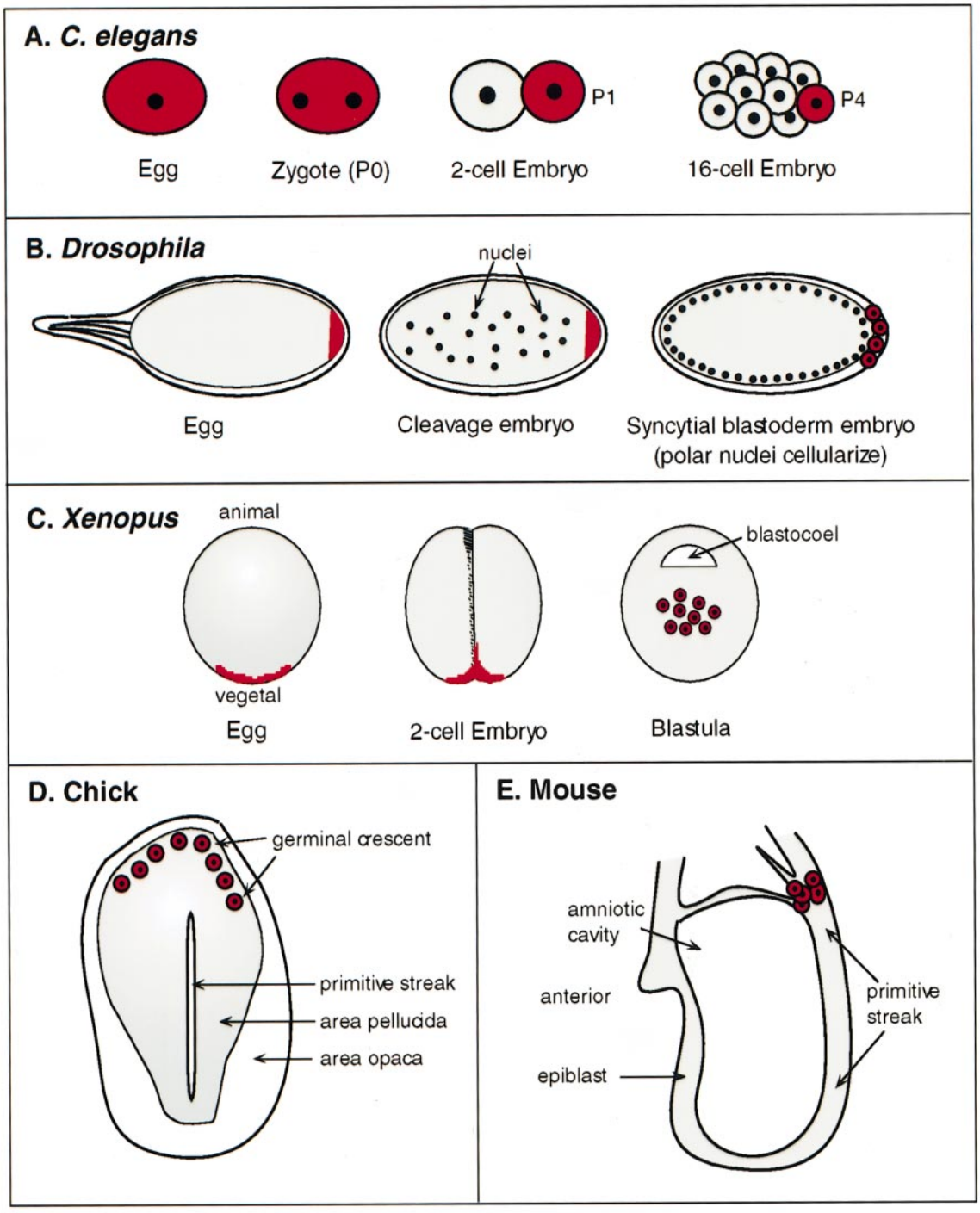

FIG. 1. Origin of PGCs. Germ plasm in A, B, and C is shown in red; PGCs in all panels are depicted in red as well. (A) *C. elegans* P granules are distributed throughout the mature egg. They are segregated away from the somatic lineage through a series of unequal divisions that produces the P1 blastomere in the 2-cell embryo and the germline founder, P4, in the 16 cell embryo. Modified from Seydoux and Strome, 1999. (B) *Drosophila* pole plasm is localized at the posterior end of the mature egg. The early embryo undergoes synchronous nuclear divisions without cytokinesis. Nuclei that arrive at the posterior end of the embryo are the first to cellularize, thus forming pole cells that include pole plasm. (C) *Xenopus* germinal plasm is localized to the vegetal pole of the mature egg. It is partitioned equally between the first four blastomeres and ingresses along the cleavage planes. At the blastula stage, germinal plasm is found in about 20 cells that are positioned near the floor of the blastocoel. Modified from Ikenishi, 1998. (D) Chick PGCs are first detected at the blastodisc stage. They are mostly found in a region called the germinal crescent that is positioned anteriorly to the embryo proper. Modified from Fujimoto *et al*., 1976. (E) Mouse PGCs can be visualized at around day 7.5 postfertilization. They are found in the area of the epiblast that is proximal to the primitive streak. Modified from Buehr, 1997.

1968). Nuage is found in female germ cells at every stage of development. In adult germ cells, nuage is associated with another germline-specific organelle called the sponge body, which consists of elongated vesicle-like elements interspersed in an electron-dense amorphous mass (Wilsch-Bräuninger *et al*., 1997). The presence of granules in pole plasm, nuage, and sponge bodies in flies could have a simple explanation: RNA-rich regions within cells appear electron-dense in electron micrographs. Thus, it has been proposed that the fibrillogranular material represents accumulated transcripts (Wilsch-Bräuninger *et al*., 1997) that change location, appearance, and probably composition as germline development proceeds.

Germinal granules in frog eggs are positioned in the vegetal cortical region of the mature unfertilized egg (Fig. 1C) within masses of mitochondria (Mahowald and Hennen, 1971; Williams and Smith, 1971). Their likely precursor is an organelle resembling sponge bodies named the mitochondrial cloud or Balbiani body, which is observed in previtellogenic oocytes of *Xenopus laevis* (Billett and Adam, 1976; Heasman *et al*., 1984; Wilsch-Bräuninger *et al*., 1997). Electron-microscopic examinations of the mitochondrial cloud have revealed a tangled network of unusually long filamentous mitochondria (Billett and Adam, 1976) within a cohesive perinuclear structure. Following fertilization, the germinal granules and the associated mitochondria are partitioned equally between the first four blastomeres. However, the third embryonic division segregates them into only four out of eight blastomeres (Akita and Wakahara, 1985; Cleine and Dixon, 1985). Subsequent asymmetric cleavages keep the germinal granules faithfully in these four cells until the 32-cell stage embryo (Ikenishi and Nakazato, 1986). At late blastula stage, 10–20 cells with germinal granules are found near the floor of the blastocoel (Blackler, 1958) and become the *Xenopus* PGCs (Fig. 1C).

Even though a *bona fide* germ plasm has not been described in the eggs of fish and birds, experimental results point to formation of PGCs during very early divisions of the zygote before embryonic cells have become committed to specific fates. In the zebrafish, *Danio rerio*, genetic studies have suggested that at the early blastula stage about five cells in the embryo are destined to give rise to the germline (Walker and Streisinger, 1983). A recently identified molecular marker for PGCs, the zebrafish homolog of the *Drosophila* gene *vasa* (discussed later), helped to identify only four cells in the 32-cell embryo as the putative founder PGCs (Amsterdam *et al*., 1997; Braat *et al*., 1999). *vasa* RNA is localized near the cortex of mature eggs (Braat *et al*., 1999), raising the possibility that some form of germline determinants, though not as distinctive as germ granules, are assembled in zebrafish eggs. Furthermore, nuagelike organelles are observed in *vasa*-positive germ cells later in zebrafish development.

The origin of PGCs in chicken has been traced in cultures of isolated fragments from early embryos (Ginsburg, 1994). As early as the blastodisc stage, a defined area constitutes the sole source of the future PGCs. Chick and quail PGCs have also been visualized directly in whole blastodisc preparations in a region called the "germinal crescent" (Fig. 1D) (Fujimoto *et al*., 1976; Yoshinaga *et al*., 1993). Recently, a chicken *vasa* homolog (*Cvh*) has been isolated and shown to have a germline-specific expression pattern (Tsunekawa *et al*., 2000). Importantly, CVH protein has been detected during very early embryogenesis starting from the first cleavage of fertilized eggs. These findings suggest that the chicken germ lineage is maternally determined and segregation of the germline occurs after the first few cleavages. As in *Xenopus*, prominent Balbiani bodies are seen in chick PGCs at later stages of development (Ukeshima and Fujimoto, 1991; see also Fig. 4).

The strategy of very early PGC determination works very well for all organisms discussed so far. The "magical" germ-cell determinants are segregated unequally during the very first embryonic divisions and the fundamental task of germ-cell establishment is accomplished. However, it is unclear whether this strategy is universal. In sea urchins, urodele amphibians (salamanders), and mammals, PGCs appear to be induced *de novo* from other cells in the early gastrulating embryo (Sutasurja and Nieuwkoop, 1974; Lawson and Hage, 1994; Ransick *et al*., 1996). In salamanders, PGCs are induced to form within the mesoderm at the time of its involution through the blastopore lips. For that reason, it has been proposed that the interaction of the dorsal endodermal cells, which pass into the interior of the embryo first, and the overlaying animal hemisphere cells creates the "right" conditions in specific involuting cells to become PGCs (Sutasurja and Nieuwkoop, 1974). Intriguingly, in the urodele amphibian axolotl, large germinal granules have been observed after fertilization in the prospective marginal zone of the embryo (Williams and Smith, 1971) where the proposed inductive interactions that lead to PGC determination should take place. This observation suggests that there may be a maternal contribution to the inductive events required for PGC formation in axolotl.

The origin of the mammalian PGCs has been studied and intensely discussed in recent years (Buehr, 1997; Matsui, 1998). In mouse embryos, each blastomere up to the eight-cell stage is equipotent (Kelly, 1977). The 16-cell embryo makes its first differentiation choice; the internal blastomeres generate the inner cell mass (ICM) that will give rise to the embryo while the external blastomeres become trophectoderm (or trophoblast cells) that will be the future placenta. After implantation into the uterine wall occurs, the ICM develops into a simple epithelium (or epiblast) that surrounds the amniotic cavity. Precursors of PGCs are localized to a region of the epiblast proximal to the primitive streak during early gastrulation [6.5 days postconception (dpc)] (Fig. 1E). Analysis of cell clones derived from a single labeled epiblast cell in the proximal region has demonstrated that the precursors of PGCs have differentiated both to PGCs and extraembryonic mesoderm (Lawson and Hage, 1994). The germline therefore has not segregated at this stage. In these experiments, the founding population

has been estimated at about 45 PGCs (Lawson and Hage, 1994). The plasticity of cell fate in the early gastrulating embryo has been further supported in transplantation experiments of *lacZ*-expressing cells to heterotopic sites in the epiblast (Tam and Zhou, 1996). Remarkably, the grafted cells followed the fate characteristic for the cells at the site of transplantation. For instance, distal cells, which normally differentiate into ectoderm, became PGCs and extraembryonic mesoderm when transplanted proximally. Thus, the hypothesis for PGC specification in mammals, as in the case of salamander's PGCs, invokes local cell interactions within the proximal epiblast or within the cells that ingress through the primitive streak at around day 7 after fertilization.

Although well supported, this belief is questioned by earlier research based on genetic mosaics (Soriano and Jaenisch, 1986; Wilkie *et al*., 1986). Mosaic animals have been created by infection of 4- to 16-cell stage mouse embryos with a recombinant retrovirus, which served as a genetic marker for the progeny of infected blastomeres (Soriano and Jaenisch, 1986). Since some proviral insertions were found either in the germline or in the somatic tissues, but not in both, the conclusion was that cells forming the germline are set aside early, prior to somatic tissue allocation. Mosaic mice also have been produced by microinjection of foreign DNA into the pronucleus of fertilized eggs (Wilkie *et al*., 1986). Analysis of these animals demonstrated that the germline sometimes entirely lacked transgenic cells while the foreign DNA was always evenly distributed among the somatic tissues. This finding supports the idea that the gemline founder pool of cells is distinct from the somatic precursor pool of cells. Hence, the question of whether mammals are dramatically different from other organisms or they follow more or less the rules of very early PGC establishment remains open.

The intrigue of the germline begins with its origin, which is not fully understood. Nevertheless, it is clear that germline segregation from the soma is one of the earliest events in development and that animals must have their PGCs established by the onset of gastrulation when dramatic movements carry them inside the embryo proper.

DETERMINATION OF PRIMORDIAL GERM CELLS DEPENDS ON LOCALIZED MOLECULES

There has long been a quest for molecules that are found or maintained only in the germline and therefore could be the germ-cell determinants. In animals with defined germ plasm, it was assumed that at least some determinants should reside in this region. Initial experiments showed that UV irradiation of amphibian and fly germ plasm produced sterile animals (Bounoure *et al*., 1954; Smith, 1966; Ikenishi *et al*., 1974; Okada *et al*., 1974). The sterility of irradiated eggs was reversed by injection of germ plasm from donor embryos that were not treated with UV light (Smith, 1966; Okada *et al*., 1974). The resulting hypothesis that nucleic acid is a possible germ-plasm component was reinforced by the finding that an electron-dense stain for RNA labeled *Drosophila* polar granules (Mahowald, 1971). A maternal RNA is critical for pole-cell formation in *Drosophila*, since injection of poly(A)$^+$ RNA from early cleavage embryos into UV-sterilized embryos led to rescue of germ-cell development (Togashi *et al*., 1986). Germ plasm in frogs also exhibits positive staining for nucleic acids, which disappears in ribonuclease-treated samples (Blackler, 1958). In *C. elegans*, P granules are likely to contain RNA as well, since clusters of poly(A)$^+$ RNAs colocalize with them starting at the 1-cell embryo (Seydoux and Fire, 1994).

In addition to RNA, isolated *Drosophila* polar granules have been shown to contain protein (Waring *et al*., 1978). Furthermore, specific germ-plasm constituents have been identified through genetic and molecular analysis of mutants. Studies of maternal-effect mutations have resulted in the identification of at least 13 fly genes required for pole-plasm assembly (reviewed in Rongo and Lehmann, 1996; Shulman *et al*., 2000). Mothers mutant for any of these genes produce embryos that lack polar granules and fail to form PGCs. Since the progeny of the mutant females are sterile, this group of mutants is called "grandchildless". Functional analyses have revealed a hierarchical relationship among these 13 gene products. Many of them localize to the posterior of the oocyte in a sequential fashion to assemble polar granules. Three of the genes, *oskar*, *vasa*, and *tudor*, play central roles in germ-plasm formation. The number of pole cells is proportional to the amount of *oskar* RNA and Oskar protein localized to the posterior pole (Ephrussi and Lehmann, 1992; Smith *et al*., 1992). Moreover, mislocalization of *oskar* RNA to the anterior pole of the *Drosophila* egg results in the assembly of ectopic polar granules and functional germ cells (Ephrussi and Lehmann, 1992). Thus, the activity of a single gene product mimics the outcome of germ-plasm transplantation at ectopic sites. Both *vasa* and *tudor* are required downstream of *oskar* for pole-cell formation at the anterior pole, and Oskar, Vasa, and Tudor proteins are all components of polar granules (Hay *et al*., 1988a; Bardsley *et al*., 1993; Breitwieser *et al*., 1996).

The precocious cellularization of pole cells in *Drosophila* embryo appears independent not only from the cellularization in the rest of the embryo, but also from pole-plasm assembly. Both the mitochondrially encoded large ribosomal RNA (mtlrRNA) and *germ cell-less* (*gcl*) RNA are pole-plasm components that are required for pole-cell formation and not for assembly of polar granules (Kobayashi and Okada, 1989; Jongens *et al*., 1992). In contrast to *oskar*, neither *mtlr* RNA nor *gcl* RNA is able to induce pole-cell formation when mislocalized (Kobayashi and Okada, 1989; Jongens *et al*., 1994). Nevertheless, embryos that express antisense *gcl* RNA have fewer pole cells, while increased levels of *gcl* RNA results in more pole cells (Jongens *et al*., 1992, 1994). Recently, characterization of a null mutation

in this gene has determined that Gcl is required for germline development (Robertson *et al*., 1999). The exact role of Gcl protein in pole-cell formation is unclear but localization to the nuclear envelope is essential.

Reduction of mtlrRNA achieved by injection of anti-*mtlr* RNA ribozymes into cleavage embryos leads to failure in pole-cell formation, even though polar granules are assembled correctly (Iida and Kobayashi, 1998). Moreover, mtlrRNA is able to rescue pole-cell formation in UV-irradiated embryos (Kobayashi and Okada, 1989). Both mtlrRNA and the mitochondrially encoded small ribosomal RNA are exported from mitochondria to polar granules before pole-cell formation and their transport is dependent on *oskar*, *vasa,* and *tudor* activities. Hence, it is speculated that mitochondrial ribosomes are present on polar granules to produce proteins required for pole-cell formation (Iida and Kobayashi, 1998; Kashikawa *et al*., 1999). Curiously, *mtlr* RNA is also enriched on germinal granules in *Xenopus* from four-cell embryo to blastula (Kobayashi *et al*., 1998), suggesting that the mtlrRNA could have a role in germline development across phylogenetic boundaries.

Today, it is well known that besides mtlrRNA, an increasing number of *Drosophila* genes that are important for germline development have homologs in other species (Table 1). Among them, Vasa, a DEAD-box RNA helicase (Hay *et al*., 1988b; Lasko and Ashburner, 1988; Liang *et al*., 1994), has been found consistently in the germline of a wide variety of animals from worms to mammals. Vasa protein is present in *Drosophila* germ cells throughout development (Hay *et al*., 1988b). Vasa is not only a polar-granule component but also is associated with the nuage, the likely precursor of pole plasm. It is thought that Vasa acts as a translational regulator of many germline-specific RNAs, thus creating and preserving at least in part the unique, "totipotent" state of germ cells. Intriguingly, a *vasa*-related gene is expressed in a totipotent somatic stem cell, the neoblast, in the flat worm *Planaria* (Shibata *et al*., 1999). The neoblast contains a chromatoid body, a structure that is similar to germ granules. After planaria are cut into small pieces, the neoblast in each one divides to regenerate the rest of the body. For this reason, it has been proposed that expression of Vasa-related proteins and/or formation of structures reminiscent of germ granules could mark a totipotent cell.

In *C. elegans*, P granules at all stages of germline development contain at least two germ-line helicases (GLHs) homologous to Vasa (Gruidl *et al*., 1996). *glh-1*, *glh-2*, or both are required for normal germline development, since injection of antisense RNA into wild-type worms results in some sterile offspring. The germline in these sterile worms is underproliferated and germ-cell nuclei have altered morphology. Importantly, P granules in mutant animals fail to stain with four out of eight monoclonal antibodies against uncharacterized P-granule epitopes (Strome and Wood, 1982; Gruidl *et al*., 1996), indicating that P-granule assembly may be defective. A third constitutive component of P granules, the PGL-1 protein, has been identified in a screen for mutants that fail to stain with monoclonal anti-P-granule antibodies. PGL-1 protein is likely to be an RNA-binding protein that shares some structural similarity to Vasa (Kawasaki *et al*., 1998). The association of PGL-1 with P granules is disrupted in *glh-1* mutants and both mutants, *pgl-1* and *glh-1*, have very similar phenotypes including germline underproliferation and absence of several P-granule epitopes. Several other proteins are localized to P granules but are also found in the cytoplasm of germ cells (see Table 1 in Seydoux and Strome, 1999). In sharp contrast to *Drosophila*, where lack of known polar-granule constituents results in failure of pole-plasm assembly, absence of any of the known worm P-granule components does not prevent their assembly. Whether P granules have a different logic of formation or simply the known P-granule constituents have later roles in granule formation remains to be shown.

Several molecules have been localized to *Xenopus* germ plasm (reviewed in Ikenishi, 1998; King *et al*., 1999; also see Table 2 in Wylie, 1999). Little, however, is known about their function in germinal-granule assembly and determination of PGCs. One of the few genes whose role in *Xenopus* germline development has been characterized is *vasa*-related, and named *Xenopus vasa-like gene 1* (*XVLG1*; Komiya *et al*., 1994). The XVLG1 protein is found in germ plasm, PGCs, and adult germ cells (Ikenishi *et al*., 1996). Unlike the *Drosophila* and *C. elegans* Vasa proteins, XVLG1 is also weakly expressed in somatic cells until young tadpole stages. XVLG1 is critical for PGC survival; when vegetal blastomeres of a 32-cell embryo are injected with anti-XVLG1 antibody and a lineage tracer, no labeled PGCs are produced at the tadpole stage (Ikenishi and Tanaka, 1997). Examination of eggs and early embryos where the maternal pool of *XVLG1* mRNA is depleted by injection of antisense deoxyoligonucleotides (Wylie and Heasman, 1997) could provide additional information about the function of XVLG1 in early germline development. At least one more Vasa-like protein is present in germ plasm and germ cells of *Xenopus* embryos (Watababe *et al*., 1992) as demonstrated by staining with a peptide antibody against the DEAD box of *Drosophila* Vasa protein. Furthermore, immunoelectron microscopy has shown that this antibody recognizes germinal granules and the matrices of mitochondria in germ cells. Cloning and functional characterization of the corresponding gene(s), however, is necessary for better understanding of the reported findings.

The molecular nature of germ-cell determinants in animals without distinctive germ plasm such as birds and mammals is still elusive. Early efforts were directed toward development of markers for PGC identification. At the time of gastrulation mammalian PGCs have been found to stain positively for the enzyme alkaline phosphatase (ALP) (EC 3.1.3.1; Chiquoine, 1954; Ginsburg *et al*., 1990), while chick PGCs are marked by the glycogen stain periodic acid-Schiff (PAS) (Meyer, 1960). Even though ALP is not specific for mammalian PGCs and a targeted disruption of the corre-

sponding gene does not affect germ cells (MacGregor *et al*., 1995), this staining has been very helpful for germ-cell identification. Starting at 8.5 dpc, individual PGCs localize ALP activity in a restricted area of the cell. This observation has led to the speculation that the enzyme is associated with a nuage-like material (Ginsburg *et al*., 1990), which, albeit inconspicuous, is present in mammalian PGCs (Eddy *et al*., 1981).

Products of *vasa* genes in both zebrafish and chicken could be germ-cell determinants. A recent study has shown that zebrafish *vasa* RNA is a component of a subcellular structure in germ cells that resembles nuage (Knaut *et al*., 2000). Curiously, although Vasa protein is in zebrafish germ cells, it is not strictly associated with nuage. In contrast, the chicken Vasa homolog is localized to granulofibrillar structures in PGCs that are in close proximity to the mitochondrial cloud (Tsunekawa *et al*., 2000). The appearance of these structures invokes a comparison with the germ plasm in organisms discussed earlier. Thus, zebrafish *vasa* RNA and chicken Vasa protein could be germ-plasm components, but whether they are required for PGC determination awaits further investigation.

In the mouse, the expression pattern of the POU transcription factor Oct-4 (also known as Oct-3; reviewed in Pesce *et al*., 1998) has suggested that this protein could be a germ-cell determinant. Oct-4 expression is gradually restricted to PGCs during mouse embryogenesis. The *oct-4* transcript is present in the egg and highly upregulated in all blastomeres of the eight-cell embryo, maintained in the ICM but downregulated in the trophoectoderm, present in the epiblast and finally restricted to just the PGCs at 8.5 dpc. Thus, Oct-4 is downregulated in all cells that will give rise to the somatic lineage but remains expressed in totipotent PGCs. Expression of a closely related homolog of Oct-4 in the urodele amphibian axolotl is also gradually confined to PGCs during gastrulation (discussed in Pesce *et al*., 1998), raising the possibility that mouse and urodele amphibians have a similar manner of PGC establishment. Unfortunately, evidence that Oct-4 is required for formation of PGCs is still lacking. Mouse embryos lacking Oct-4 due to a targeted gene deletion die at a blastocyst stage prior to the earliest detection of PGCs (Nichols *et al*., 1998). A conditional knockout using the Cre/loxP system that could remove Oct-4 at gastrulation or a more subtle gene disruption could assess whether PGCs form in the absence of this molecule.

The hypothesis that a signaling event induces cells within the proximal epiblast to become the mammalian PGCs has gained molecular support from studies of mouse embryos deficient for the Bone Morphogenetic Protein 4 (BMP4), a TGFβ family member (Lawson *et al*., 1999; McLaren, 1999). The homozygous mutant embryos completely lack PGCs while the heterozygous siblings have fewer PGCs than wild-type embryos. A similar phenotype has been described recently in mouse embryos that are homozygous or heterozygous for a null mutation in the gene encoding BMP8b (Ying *et al*., 2000). BMP4 and BMP8b are found at the right time and place; they are expressed before gastrulation in the extraembryonic ectoderm with highest levels at the junction with the proximal epiblast. Whether or not BMP4 and BMP8b are the direct signals for determination of mammalian PGCs remains to be shown. However, a requirement for the presence of these two proteins appears well established. Intriguingly, the *Drosophila* counterpart of BMP4, Decapentaplegic (DPP), is essential for maintenance of germline stem cells in the adult ovary (Xie and Spradling, 1998). In this case, the signaling molecule is most likely secreted from the surrounding somatic cells and acts directly on germline stem cells to maintain their ability for self-renewal. Thus, both the mouse and fly germ cells respond to BMP4 signaling and must express the appropriate receptors and downstream effector molecules.

The number of molecules that have been localized to PGCs in different animal species is growing continuously. Some of these molecules are *bona fide* germline determinants. Some of them appear species-specific; for example, apparent homologs of fly *oskar* and worm *pgl-1* have not been characterized in other organisms so far. At the same time, the finding that genes important for *Drosophila* germline development are evolutionarily conserved raises the possibility for common molecular mechanisms of germline establishment. Providing evidence for functional conservation will be a major challenge in future research.

PRIMORDIAL GERM CELLS ACTIVELY MAINTAIN THEIR IDENTITY

C. elegans and Drosophila PGCs Control the Onset of Their Zygotic Transcription

Once formed, the identity and developmental potency of the germline is maintained throughout the life of the animal. This ability is especially important for PGCs immediately after their determination; as suggested many years ago, PGCs must have a way to keep their identity when the rest of the embryonic cells commence a very active process of somatic differentiation (Blackler, 1958). Recent studies in *C. elegans* and *Drosophila* have indicated that PGCs shield themselves mainly through transcriptional repression of their own genome (reviewed in Seydoux and Strome, 1999). This way germ cells delay the initiation of zygotic transcription that otherwise would promote a somatic cell fate.

Transcriptional patterns differ significantly between PGCs and somatic blastomeres in both worm and fly embryos. While newly transcribed mRNAs can be detected in somatic nuclei as early as four-cell worm embryos, transcriptional activity in the germline precursors, Z2 and Z3, begins around the 100-cell stage (Seydoux *et al*., 1996; Seydoux and Dunn, 1997). Similarly, zygotic transcription in *Drosophila* somatic cells is detected as early as 1 h after egg laying (ael) (Zalokar, 1976; Pritchard and Schubiger, 1996). In contrast, general mRNA synthesis in fly germ

TABLE 1
Conserved Genes in Germ-Cell Development

Gene (Protein)	Species	Localization, function	References
vasa (RNA helicase)	*Drosophila*	Present on pole granules; translational regulation, pole-plasm assembly	Hay *et al.*, 1988b; Lasko and Ashburner, 1988; Liang *et al.*, 1994
	C. elegans (glh-1 & glh-2)	Present in PGCs	Roussell and Bennett, 1993; Grundl *et al.*, 1996
	Planaria	Present in neoblast	Shibata *et al.*, 1999
	Xenopus (XVLG1)	Present in PGCs	Komiya *et al.*, 1994
	Zebrafish	Present in PGCs	Yoon *et al.*, 1997; Braat *et al.*, 1999
	Rainbow trout	Present in PGCs	Yoshizaki *et al.*, 2000
	Chicken (*CVH*)	Present in PGCs	Tsunekawa *et al.*, 2000
	Mouse (*MVH*)	Present in PGCs	Fujiwara *et al.*, 1994
	Rat	Present in PGCs	Komiya and Tanigawa, 1995
	Human	Present in PGCs	Castrillon *et al.*, 2000
mago nashi (novel)	*Drosophila*	Pole-plasm assembly	Newmark and Boswell, 1994
	C. elegans	Rescues *Drosophila mago* mutant; role in sex determination	Newmark *et al.*, 1997; Li *et al.*, 2000
	Xenopus		Newmark *et al.*, 1997
	Mouse		Newmark *et al.*, 1997
	Human		Zhao *et al.*, 1998
tudor (novel, ten tudor domains)	*Drosophila*	Component of pole granules; pole-plasm assembly	Golumbeski *et al.*, 1991
	Human (proteins with single tudor domains)	Nuclear	Callebaut and Mornon, 1997; Buhler *et al.*, 1999
germ-cell less (nuclear protein)	*Drosophila*	Associated with nuclear pores; pole-cell cellularization	Jongens *et al.*, 1992; 1994
	C. elegans		Kimura *et al.*, 1999
	Mouse	Present in PGCs; implicated in cell-cycle control	de la Luna *et al.*, 1999; Kimura *et al.*, 1999
mtlrRNA (mitochondrially encoded large ribosomal RNA)	*Drosophila*	Formation of mitochondrial ribosomes on pole granules	Kobayashi and Okada, 1989; Iida and Kobayashi, 1998
	Xenopus	Present in PGCs	Kobayashi *et al.*, 1998
nanos (RNA-binding protein)	*Drosophila*	Present in pole plasm and PGCs; translational regulation	Wang and Lehmann, 1991; Parisi and Lin, 2000
	C. elegans (three related genes)	Present in PGCs; gonad formation and germ cell maintenance, sperm-oocyte switch	Subramsniam and Seydoux, 1999; Kraemer *et al.*, 1999
	Xenopus (Xcat2)	Present in germinal plasm and PGCs	Mosquera *et al.*, 1993
	Leech		Pilon and Weisblat, 1997
pumilio (RNA-binding protein)	*Drosophila*	Present in pole plasm and PGCs; translational regulation	Macdonald, 1992; Parisi and Lin, 2000
	C. elegans (eight related genes)	Present in PGCs; gonad formation and germ-cell maintenance	Subramaniam and Seydoux, 1999
	Human	Shows specificity of RNA binding related to fly Pumilio	Zamore *et al.*, 1997

cells is not observed until 3.5 h ael (Zalokar, 1976). RNA polymerase II (RNAP II), the enzyme responsible for mRNA synthesis, is present in both somatic and germline nuclei in *C. elegans* and *Drosophila* early embryos (Seydoux and Dunn, 1997). However, the germline nuclei in pregastrulating embryos lack a subpopulation of phosphorylated RNAP II. The missing phosphoepitope maps to the carboxy-terminal domain (CTD) of the large subunit of RNAP II and is critical for the transition from the initiation to the elongation phase of transcription (reviewed in Dahmus, 1996). Importantly, CTD-phosphorylated RNAP II appears in germ cells coincidentally with the onset of zygotic transcription in these cells. Unlike mRNA synthesis, production of rRNA is readily detectable in PGCs of both worm and fly early embryos, indicating that RNA polymerase I remains active (Seydoux and Dunn, 1997).

In *C. elegans*, a maternally expressed gene, *pie-1*, has been shown to play a central role in transcriptional repression in germline blastomeres. In embryos from *pie-1* mutant females, the germline blastomere P2 adopts a somatic cell fate, producing additional pharyngeal and intestinal cells (Mello *et al*., 1992). Thus, these embryos lack the germline lineage, even though the initial segregation of P granules is normal. The hypothesis that PIE-1 protein is responsible directly for the inhibition of germline transcription is supported in several ways. First, *pie-1* embryos transcribe prematurely several mRNAs in early germ cells (Seydoux *et al*., 1996). Furthermore, the phosphorylated-on-CTD RNAP II is expressed earlier as well, in a pattern identical to the observed in somatic cells (Seydoux and Dunn, 1997). Second, PIE-1 is present only in germline blastomeres where it is found in the cytoplasm and the nucleus (Mello *et al*., 1996; Tenenhaus *et al*., 1998). Last, PIE-1, a protein with two zinc fingers, directly represses transcription in cell-culture transcriptional assays most likely through interference with a protein that recognizes the CTD of RNAP II (Batchelder *et al*., 1999). Besides inhibition of transcription, PIE-1 could be involved in regulation of translation of maternally deposited RNAs; at least one protein, NOS-2 (discussed below), fails to be translated in the germline founder P4 in *pie-1* mutant embryos (see Seydoux and Strome, 1999).

PIE-1 protein is not expressed in germ cells past 100-cell embryo; nevertheless, the PIE-1 "legacy" is maintained and the germline keeps its distinctive characteristics. At least four maternal-effect genes, *mes-2*, *mes-3*, *mes-4*, and *mes-6* (*mes* = maternal effect sterile), are required for this later step of germline maintenance. These genes have been isolated in a grandchildless screen where homozygous mutant mothers are fertile but produce sterile progeny (Capowski *et al*., 1991). Newly hatched larvae from mothers mutant in any of these four genes have germ cells; however, proliferation is limited and a progressive degeneration of germ cells is observed later in development. An insight into the function of the *mes* genes has come from the molecular identity of their gene products. Both *mes-2* and *mes-6* encode proteins homologous to the members of the *Drosophila* Polycomb group (Pc-G) of transcriptional repressors (reviewed in Pirrotta, 1997). MES-2 is homologous to Enhancer of Zeste, while MES-6 is related to Extra Sex Combs (Holdeman *et al*., 1998; Korf *et al*., 1998). Members of the Pc-G in *Drosophila* form multimeric protein complexes that associate with chromatin to modify its structure at distinct chromosomal sites. This chromatin modification results in transcriptional silencing of certain genes such as the homeotic gene complexes. These findings have fostered the hypothesis that MES proteins in *C. elegans* function in a context-dependent fashion to modulate chromatin structure and gene expression in the early germline. Further support of this idea has come from studies on silencing of transgenic DNA in the worm germline (Kelly and Fire, 1998). Transgenes in *C. elegans* are generally transmitted as many tandem copies (or arrays) of the injected DNA. Although expressed in somatic lineages, the transgenic arrays are silenced in the worm germline. This phenomenon is abolished in a *mes* mutant background and the tested transgenic array is expressed in the residual germ cells (Kelly and Fire, 1998). Thus, MES proteins are likely to maintain germline identity by silencing somatic-specific transcription while allowing expression of genes necessary for further germ-cell differentiation. At least one gene from the Pc-G, *polycombeotic*, is known to cause female sterility and/or maternal effect lethality in flies (Phillips and Shearn, 1990). Whether this phenotype is due to inappropriate gene expression in the germ cells of adult ovaries and early embryos has not been investigated.

Transcriptional Repression in Drosophila Pole Cells Depends on the Translational Regulators Nanos and Pumilio

The establishment and maintenance of transcriptional quiescence in *Drosophila* PGCs is mediated by Nanos and Pumilio, two proteins that are incorporated in pole cells as they form. *nanos* RNA is localized to the posterior pole of the egg and this localization depends on *oskar*, *vasa*, and *tudor* activities. Nanos protein is translated early in embryogenesis from this localized source to create a posterior-to-anterior protein gradient. In addition to a role in germline development, Nanos is required for correct abdominal patterning of the *Drosophila* embryo. To accomplish the latter function, Nanos together with Pumilio protein represses the translation of maternal *hunchback* mRNA, thereby allowing proper abdominal development (reviewed in Rongo *et al*., 1997).

nanos and *pumilio* are dispensable for pole-plasm assembly and pole-cell formation in *Drosophila*. However, many subsequent events of germ-cell development, such as migration of PGCs to the primordial somatic gonad and oogenesis in adults, require *nanos* and *pumilio* activities (Kobayashi *et al*., 1996; Forbes and Lehmann, 1998; Asaoka-Taguchi *et al*., 1999). Notably, PGCs lacking maternally deposited Nanos or Pumilio proteins prematurely activate several germline transcriptional reporters (Kobayashi *et al*., 1996; Asaoka-Taguchi *et al*., 1999). Furthermore, genes that are normally active in the soma but not in the germline of early embryos are transcribed in *nanos* mutant pole cells (Deshpande *et al*., 1999). Therefore, Nanos is responsible for a more general regulation of transcriptional repression in early germ cells, invoking a comparison with the function of PIE-1 protein in early *C. elegans* embryo. In contrast to PIE-1, however, there is no evidence so far that Nanos directly represses transcription. Instead, Nanos is an RNA-binding protein capable of repressing translation of certain mRNAs and thus could influence transcriptional activity indirectly. Also, unlike absence of PIE-1, lack of Nanos does not affect the phosphorylation profile of RNAP II, suggesting that Nanos regulates gene expression after general transcription is activated in the germline (Seydoux and Dunn, 1997). Hence, Nanos is not functionally homologous

to PIE-1, and a *Drosophila* PIE-1 homolog still awaits discovery. Although three *nanos*-related genes are present in *C. elegans* and they are important for germ-cell development (Kraemer *et al.*, 1999; Subramaniam and Seydoux, 1999; and discussed later), their participation in transcriptional repression in early embryos has not been defined thus far.

C. elegans and Drosophila PGCs Regulate Their Cell Cycle

Another feature that distinguishes fly and worm PGCs from somatic cells is their relative mitotic inertness. In *Drosophila*, pole cells cease mitosis at gastrulation and remain mitotically arrested until they reach the primordial somatic gonad. It is thought that the arrest in the G2 phase of the cell cycle (Su *et al.*, 1998) is another protective mechanism that prevents dilution of maternal factors incorporated in pole cells. Thus, factors like Nanos and Pumilio would be kept in high enough concentrations to maintain germline identity and be available for pole-cell migration later (see Asaoka-Taguchi *et al.*, 1999). The very same proteins seem to regulate this mitotic arrest; pole cells devoid of either maternal Nanos or Pumilio enter mitosis prematurely (Asaoka-Taguchi *et al.*, 1999; Deshpande *et al.*, 1999). A plausible target of Nanos and Pumilio is *cyclin B* RNA, which is localized to germ plasm and partitioned into pole cells but not translated until late in embryogenesis (Dalby and Glover, 1993). Indeed, Cyclin B is precociously expressed in pole cells lacking Pumilio or Nanos, suggesting that these two proteins could directly repress *cyclin B* RNA translation, thereby inhibiting the transition from G2 phase to mitosis in wild-type pole cells (Asaoka-Taguchi *et al.*, 1999).

In *C. elegans*, the PGCs Z2 and Z3 have the ability to regulate their cell cycle as well. Z2 and Z3, together with other stem cells, cease cell division until the larva begins to feed. Starved larvae maintain a block in cell proliferation and survive for several days. In contrast, Z2 and Z3 initiate premature proliferation in starved larvae that are doubly mutant for *nanos-1* and *nanos-2* (Subramaniam and Seydoux, 1999). Similar observations have been made when five of the eight *pumilio*-related genes in *C. elegans* have been simultaneously disrupted using RNA-mediated interference (Subramaniam and Seydoux, 1999). The mechanism of cell-cycle regulation in the worm germline by *nanos*- and *pumilio*-related genes has not been clarified. An attractive hypothesis is that Nanos and Pumilio proteins in worms could be controlling translation of important cell-cycle regulators, as they do in flies.

The strategies to maintain germline identity are remarkably similar in *Drosophila* and *C. elegans*. Moreover, related proteins in flies and worms lead to almost identical phenotypes when absent from PGCs, suggesting that molecular mechanisms could be conserved as well. Genes homologous to *nanos* and *pumilio* are also found in other animals, including *Xenopus* and humans (see Table 1) but functional conservation has yet to be demonstrated.

Methylation Controls Gene Expression in Mammalian PGCs

In mammals, zygotic transcription begins very early in embryogenesis. Embryos transcribe their own genes starting at the two-cell stage in mouse and the eight-cell stage in human (reviewed in Schultz, 1993). Although direct evidence is lacking, it is likely that the phenomenon of delayed RNAP II-dependent transcription in PGCs does not exist in these animal species. Instead, at the time of activation of zygotic transcription, a very active process of DNA demethylation occurs. It has been hypothesized that this demethylation removes the differences in gene methylation patterns that emerge from the male and female gametes, thereby allowing initiation of a normal pattern of embryonic development (Kafri *et al.*, 1992). DNA methylation is known to have a profound effect on both chromatin structure and gene expression. Specifically, methylation represents one of the cellular mechanisms for suppression of gene activity. Mouse embryos undergo dynamic changes in methylation pattern and the initial global demethylation is followed by almost ubiquitous remethylation prior to gastrulation (6.5 dpc), which subsequently is subjected to tissue-specific demethylation events. Fetal germ cells, however, exhibit a significant delay in this genome-wide remethylation; they are completely unmethylated at 12.5 dpc (Monk *et al.*, 1987), partially methylated at 15.5 dpc, and completely remethylated by 18.5 dpc (Kafri *et al.*, 1992). Hence, it has been proposed that PGCs delay *de novo* methylation to preserve the totipotency of the early embryonic cells. This delay in remethylation could be viewed as analogous to the delay in zygotic transcription that is observed in fly and worm PGCs; in either case, PGCs avoid the dramatic changes that occur in somatic cells of early embryos and maintain their initial identity.

The global methylation in differentiating somatic cells could result in downregulation of Oct-4 expression. Changes in both chromatin structure and methylation of the 5′ upstream region have been implicated in the extinction of Oct-4 transcription (Ben-Shushan *et al.*, 1993). Since the early mammalian PGCs are completely unmethylated, Oct-4 should not be silenced and should be able to function not only in the formation of PGCs but also in maintenance of germline identity.

A second round of demethylation in mammalian germ cells is sex- and gene-specific and, in the female, occurs during oocyte maturation (Kafri *et al.*, 1992). Therefore, germline-specific transcription seems to be monitored in a context-dependent manner in later stages of mammalian germ-cell development. This kind of localized regulation of gene expression is reminiscent of the germ-cell specific transcriptional regulation by the Pc-G genes in worms.

Thus, evolutionarily distant animal classes such as invertebrates and mammals use similar strategies to preserve the unique state of germ cells. Even though the specific molecular mechanisms appear quite divergent, the transcriptional regulation is accomplished by functionally similar epigenetic modifications.

GERM CELLS HAVE AN EXTRAGONADAL ORIGIN AND MIGRATE TO REACH THE SOMATIC GONAD

Comparative Aspects of Migratory Movements in Different Animal Species

Germ cells rarely differentiate into gametes at the place where they first appear. Instead, in most metazoans PGCs search out special somatic cells with which to form the gonad. PGCs are first discernible at the margin of the embryo proper. As gastrulation proceeds, germ cells are carried rather passively inside the embryo where they are found in close association with the developing gut. From there, PGCs navigate through different tissues to recognize and unite with the somatic gonadal primordium.

The movements that lead fly and mammalian PGCs to the prospective somatic gonad are highly stereotypical and remarkably similar. Pole cells in *Drosophila* (Fig. 2A, arrowhead) enter the embryo (Fig. 2B) together with endodermal cells that give rise to the posterior midgut (PMG) (reviewed in Williamson and Lehmann, 1996). Similarly, mammalian PGCs lie in close proximity to the hindgut and are embedded in the endodermal gut wall as it forms (reviewed in Buehr, 1997; Fig. 2G; 9.5 dpc). Both mammalian and fly PGCs then actively migrate through the gut epithelial layer. They develop large pseudopodia (Clark and Eddy, 1975; Warrior, 1994) that scan in various directions until finding large intercellular spaces in the epithelial wall (Callaini *et al*., 1995; Jaglarz and Howard, 1995). Having traversed the gut wall, *Drosophila* pole cells continue to move dorsally through the endoderm and come in contact with the overlaying mesoderm (Fig. 2C). At this stage two groups of mesodermal cells are easily distinguishable by the transcription from a specific transposon (a retrovirus-like element called 412) and represent the precursors of the somatic gonad (Brookman *et al*., 1992). They lie symmetrically on either side of the embryonic midline and are reached by 10–15 pole cells (Fig. 2D). Pole cells align with the somatic gonadal precursors (Fig. 2E) and finally coalesce with them to form the embryonic gonad (Fig. 2F).

Having left the gut wall, the mammalian PGCs also travel up into the dorsal mesentery, which connects the developing gut to the dorsal body wall (Fig. 2G; 10.5 dpc). PGCs then split into two groups that migrate laterally to arrive to the somatic gonadal primordia (or genital ridges), which develop on either side of the dorsal aorta. The steps of PGC migration in *Xenopus* closely resemble those described for mammalian PGCs (Wylie and Heasman, 1976; Heasman and Wylie, 1981). Zebrafish PGCs also have characteristics similar to migrating PGCs of *Drosophila* and mammals, such as association with the endoderm and the dorsal aspect of the gut, formation of long pseudopodia (Fig. 2H), and coalescence with two groups of somatic gonadal cells (Braat *et al*., 1999).

As migration proceeds, mouse germ cells establish contacts not only with somatic cells but also with each other; long filopodial processes have been observed to link them in extensive networks (Gomperts *et al*., 1994). Subsequently, these linked germ cells aggregate and form compact clusters within the genital ridges. Similar interaction between PGCs through extended processes occurs in the *Drosophila* embryo that ends as germ cells coalescence with the gonadal mesoderm (Jaglarz and Howard, 1995).

Germ cells in *C. elegans* and birds possess some unique features in gonad formation. After entering the gastrulating worm embryo, the P4 cell gives rise to Z2 and Z3, which extend protrusions into the gut primordium and toward each other (Sulston *et al*., 1983). Z2 and Z3 then remain rather static while the somatic gonadal cells migrate toward them. In birds, most PGCs migrate using the circulatory system (Meyer, 1964; Yoshinaga *et al*., 1993). These circulating PGCs (cPGCs) are round, exhibit a relatively smooth surface, and are carried to the vicinity of the genital ridges where they exit the bloodstream and actively migrate to colonize the somatic gonad (Yoshinaga *et al*., 1993). The passage of cPGCs through the blood vessel wall is similar to the migration of mouse and *Drosophila* PGCs through the gut epithelium. cPGCs must "sense" precisely where to exit the capillaries but the mechanism of this event is not understood. Also, entry in the bloodstream is not obligatory since a small fraction of avian PGCs never go into the blood vessels and reach the somatic gonad with ameboid movements (Fujimoto *et al*., 1976).

Mitotic activity of PGCs in the course of migration varies among species. Mammalian PGCs actively divide as they migrate. In the mouse, the approximate doubling time of a germ cell is 16 h and the number of PGCs increases from 145 at 8 dpc to about 3000 at 11.5 dpc when migration ends (Tam and Snow, 1981). In contrast, PGCs in several other species are largely quiescent during migration. Germ cells in *Xenopus* and zebrafish undergo about three division cycles, such that 25 to 30 PGCs populate the gonads (Gomperts *et al*., 1994; Braat *et al*., 1999). PGCs in *Drosophila* and *C. elegans* are also mitotically inactive during embryogenesis and resume divisions in larvae; *Drosophila* pole cells divide up to 2 times after cellularization while *C. elegans* P4 divides just once to produce Z2 and Z3 (Sonnenblick, 1950; Sulston *et al*., 1983).

Genes and Mechanisms Involved in PGC Migration

The germ-cell journey through the embryo is carefully orchestrated; although a few are lost, most PGCs successfully reach their final destination. Currently, identification of molecules that are necessary for migration is an area of active research. The findings indicate that the germline and

soma cooperate in gonad formation: the PGCs are not simply passengers and neither is the somatic gonadal primordium merely the waiting destination.

At present, PGC migration is best characterized molecularly in *Drosophila*. In genetic screens, a number of somatically expressed genes have been identified with distinct contributions to discrete steps of the migration process (Warrior, 1994; Boyle and DiNardo, 1995; Zhang *et al*., 1996; Boyle *et al*., 1997; Moore *et al*., 1998). Two genes, *wunen* and *columbus*, are involved in the production of guidance cues. Wunen provides as a repulsive signal that excludes migrating pole cells from inappropriate places (Zhang *et al*., 1997). In contrast, *columbus* is required for the production of a factor in the gonadal mesoderm that attracts pole cells (Van Doren *et al*., 1998). When misexpressed, *columbus* is sufficient to attract PGCs to tissues other than the gonadal mesoderm; conversely, misexpressed *wunen* transforms a tissue that is normally permissive to migrating PGCs into a repulsive one. Although these two genes are clearly important for PGC migration, their molecular identities do not provide an immediate explanation for their function. Wunen protein is related to phosphatidic acid phosphatase 2 (PAP-2; Zhang *et al*., 1997), an enzyme that spans the plasma membrane 6 times. Thus, this protein could serve directly as a repellent. Alternatively, a product of phospholipid metabolism could be the repulsive factor or could be responsible for the production of this factor. The *columbus* gene encodes the *Drosophila* homolog of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that is involved in cholesterol biosynthesis in humans (Van Doren *et al*., 1998). Since flies do not make cholesterol, another lipid product (e.g., a terpene derivative) could be the attractive cue. It is also possible that a modification of an unknown protein could be the final outcome of the *columbus* function. Identification of additional mutants with similar phenotypes combined with further functional studies of *wunen* and *columbus* are needed to address the current models.

Germ cells in *Drosophila* recognize the somatic guidance cues and correctly interpret them. The identity of the germ-cell receptors and downstream signaling cascades are largely unknown. Two genes discussed earlier, *nanos* and *pumilio*, are required in pole cells for their successful migration to the somatic gonad. Absence of maternally provided Nanos or Pumilio results in pole cells that traverse the gut wall but stall at the outer gut surface (Kobayashi *et al*., 1996; Forbes and Lehmann, 1998; Asaoka-Taguchi *et al*., 1999). Unlike wild-type pole cells, which are large and have a smooth surface, *nanos* pole cells are of varying size and often have an irregular surface (Forbes and Lehmann, 1998). These mutant pole cells cluster tightly together as soon as they leave the gut epithelium, a behavior that is characteristic for pole cells that are already associated with the gonadal mesoderm. For that reason, it is proposed that *nanos* and *pumilio* pole cells differentiate prematurely and act as if they have completed migration to the somatic gonads (Forbes and Lehmann, 1998; Asaoka-Taguchi *et al*., 1999). The untimely differentiation and migration failure could be a result of precocious gene expression that is observed in both *nanos* and *pumilio* mutant germ cells (discussed earlier). One of the recently discovered Nanos targets is the RNA-binding protein Sex lethal (Sxl), which functions as both a splicing and a translational regulator (Deshpande *et al*., 1999). Removal of the *Sxl* gene attenuates the migration defects of *nanos* mutant pole cells, while ectopic Sxl expression causes migration problems and changes in pole-cell morphology that are very similar to those observed in a *nanos* mutant background.

The roles of *nanos* and *pumilio* in gonad assembly appear conserved in at least one more species. When expression of *nanos-2* or five *pumilio*-like genes is prevented, one of the PGCs in *C. elegans* is found consistently outside of the gonad. *nanos-2* and a subset of *pumilio*-related genes, therefore, are required for efficient incorporation of the worm PGCs into the somatic gonad (Subramaniam and Seydoux, 1999).

Completion of germ-cell migration depends on the presence of specific germ-plasm components. In *Drosophila*, a noncoding RNA, called *Polar granule component* (*Pgc*), has an essential role in later stages of pole-cell migration (Nakamura *et al*., 1996). Pole cells in embryos that express antisense-*Pgc* show severe defects in their association with the somatic gonadal precursors and fail to populate the embryonic gonads. In *Xenopus*, a germ-plasm RNA encoded by the *Xdazl* gene is required for the exit of germ cells from the endoderm (Houston and King, 2000). *Xdazl* is related to the genes of the *Deleted in Azoospermia* (*DAZ*) family, which are RNA-binding proteins with a role in germline development in a number of organisms. Antisense-mediated depletion of the maternal *Xdazl* RNA results in clusters of PGCs in the endoderm that never enter the dorsal mesentery. Thus, germ-plasm constituents in flies and frogs are required not only for the initial specification of PGCs but also for the successful incorporation of determined germ cells into the somatic gonads.

Similar to pole cells in *Drosophila*, migrating PGCs in the mouse interact with the surrounding somatic tissues. Mechanisms that guide mouse germ cells, such as attraction by the developing gonad and contact with the extracellular matrix (ECM) proteins, and several participating molecules have been studied over the years (reviewed in Buehr, 1997). It has been demonstrated that genital ridges release a chemotropic factor(s) that stimulates germ-cell proliferation and attracts PGCs toward them (Rogulska *et al*., 1971; Godin *et al*., 1990). Also, an interaction of PGCs with ECM molecules has been examined (De Felici and Dolci, 1989; Garcia-Castro *et al*., 1997) and recently supported genetically (Anderson *et al*., 1999). Germ cells express a number of integrin subunits, which as heterodimers could mediate an attachment to the ECM molecules laminin and fibronectin. Examination of animals that are chimeric for *integrin* $\beta 1$ revealed that *integrin* $\beta 1^{-/-}$ PGCs do form. However, these mutant PGCs stall in the gut endoderm and do not colonize the

somatic gonads (Anderson *et al.*, 1999). The role of laminin, which is the ligand for integrin β1, is still awaiting genetic investigation in mouse germ-cell migration. It is already known, though, that laminin A is required *in vivo* for the migration of pole cells in *Drosophila* and this protein also supports motility of isolated pole cells *in vitro* (Jaglarz and Howard, 1995).

The ability of PGCs to adhere must be carefully controlled so that they do not attach prematurely to tissues along their migratory pathway. A candidate for a molecule that can confer antiadhesive properties in chick and rat PGCs is ovomucin. Ovomucin is a cell-surface glycoprotein that is expressed on migratory PGCs but not in germ cells that have already settled in the gonad (Halfter *et al.*, 1996). *In vitro* this protein is a poor substratum for adhesion of somatic mesenchymal cells. Thus, it has been hypothesized that ovomucin is needed to prevent precocious adhesion of migrating PGCs to blood-vessel walls and to connective tissue in the mesentery. Therefore, a balance between the competence of PGCs to adhere or not to adhere to surrounding somatic tissues may be crucial for their movement.

Migrating mouse PGCs need the c-Kit receptor protein expressed on their surface and its ligand, the Steel factor (or Stem Cell Factor) present on the neighboring somatic cells for successful colonization of the gonadal ridges. Strong mutations in the *W* locus (*Dominant White spotting*), which encodes the receptor tyrosine kinase c-Kit, or in the *Steel* locus (producing the Steel factor) manifest nearly identical defects in gametogenesis, melanogenesis, and hematopoiesis (Besmer *et al.*, 1993). PGCs in these mutants form normally; however, they fail to proliferate, clump together prematurely, and are often found at ectopic sites (Buehr *et al.*, 1993). As a result, very few germ cells populate the genital ridges and the adults are sterile. At present, it is unclear whether these two genes function in several germ-cell events or failure in one process prevents correct performance afterwards. In wild-type mice, PGCs express the c-Kit receptor during migration when they also proliferate (Manova and Bachvarova, 1991). Intriguingly, the somatic cells along the germ-cell migratory pathway transcribe the mRNA for the Steel factor and highest levels of this RNA are observed in the developing gonad (Keshet *et al.*, 1991). Thus, a continuous interaction between germline and soma could occur and a gradient of Steel protein could serve as a homing mechanism for the migrating PGCs. This idea is supported by cell-culture experiments in which adhesion of PGCs to somatic cells is significantly reduced by antibodies to either the c-Kit receptor or the Steel factor (Pesce *et al.*, 1997).

In conclusion, germ cells in divergent animal classes have strikingly similar itineraries as they navigate toward the somatic gonad. The mechanisms of migration, involving the use of intrinsic and somatic cues, attraction and repulsion, and ameboid motility, also appear highly conserved. Most of the participating molecules, however, have a role in

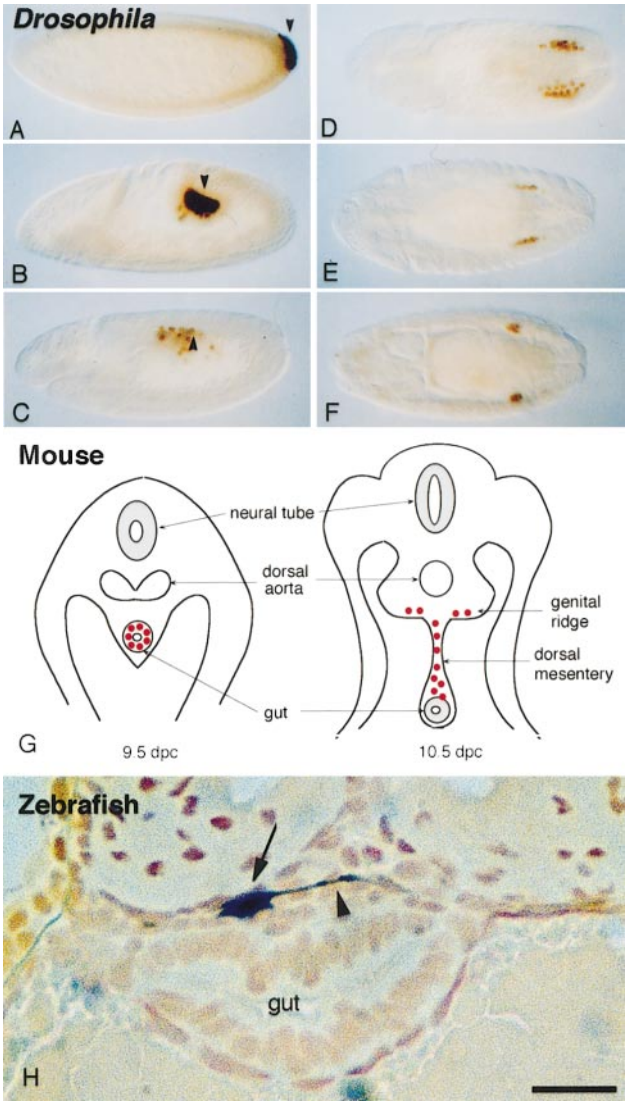

FIG. 2. Germ-cell migration in *Drosophila*, mouse, and zebrafish. (A–F) *Drosophila*. Germ cells are visualized with anti-Nanos antibody. A–C show lateral views while D–F are dorsal views of embryos. (A) Germ cells form at the posterior end of the embryo. (B) Germ cells are carried inside the embryo during gastrulation and are found in the posterior midgut lumen. (C) Germ cells migrate dorsally through the wall of the posterior midgut. (D) Germ cells associate with the somatic gonadal precursors. (E) All germ cells align with the somatic gonadal mesoderm. (F) The germ cells and the somatic gonadal mesoderm coalesce to form the embryonic gonad. Adapted from Moore *et al.*, 1998. (G) Mouse. At 9.5 dpc mouse PGCs are embedded in the gut. At day 10.5 the PGCs have left the gut and are moving up through the dorsal mesentery to reach the paired genital ridges. Adapted from Buehr, 1997. (H) Zebrafish. This zebrafish germ cell (arrow) is visualized with anti-Vasa antibody. The migrating PGC is found at the dorsal aspect of the gut extending a pseudopodium (arrowhead) that is 1.5 times longer than the cell body. Scale bar, 20 μm. Reproduced from Braat *et al.*, 1999.

gonad assembly in individual species that has not been extended to other organisms at this time.

GERM CELLS BEGIN GONADAL DEVELOPMENT AS CLUSTERS OF INTERCONNECTED CELLS

Once the gonad is assembled, germ cells begin active proliferation and become gonial cells. In many invertebrate and vertebrate organisms, single female gonial cells, also known as oogonia, divide to form clusters of interconnected cells (reviewed in Pepling *et al*., 1999). The founder cell is called a cystoblast, while the cluster is designated as a cyst. As a result of synchronized divisions, cell number in the cyst frequently corresponds to powers of 2. Usually, the cells in the cyst do not complete cytokinesis and remain joined via stable intercellular bridges to create a syncytium. Male gonial cells also have a syncytial organization, but spermatogenesis will not be covered in this review.

Syncytial Development of Drosophila and C. elegans Oogonia

During *Drosophila* oogenesis, each cystoblast undergoes four consecutive mitotic divisions to give rise to a cyst of 16 germline cells (Fig. 3A) that persists until late stages of oogenesis. Only one cell develops as an oocyte within the cyst; the other 15 cells differentiate into nurse cells whose main function is to supply the growing oocyte with nutrients. The cyst breaks down toward the end of oogenesis when a concerted cellular suicide removes the spent nurse cells. As in other animal species, the intercellular bridges within a *Drosophila* cyst originate from arrested cleavage furrows (reviewed in Mahajan-Miklos and Cooley, 1994). The shape of the intercellular bridge and the frequent finding of microtubules within its lumen have suggested that cytokinesis could be halted due to the presence of a persistent midbody. A characteristic feature of all described stable intercellular bridges is a thickened, electron-dense plasma membrane (see Fig. 3C). *Drosophila* intercellular bridges, also known as ring canals, become reinforced with an additional inner layer. This inner layer is composed of filamentous actin and several other proteins that are required for structural integrity and growth (reviewed in Cooley, 1998). Ring canals gradually expand in diameter from 0.5 to about 10 μm to accommodate the constant stream of cytoplasm and organelles from the 15 synthetically active nurse cells to the developing oocyte.

The formation of a 16-cell cyst in *Drosophila* depends on the presence of the fusome, a germline-specific organelle that is rich in small ER-like vesicles (reviewed in de Cuevas *et al*., 1997). A fusome is present in the cystoblast and subsequently grows in a highly stereotypical manner to form branches that extend through the ring canals of the 16-cell syncytium. The fusome branches come in contact with one pole of the mitotic spindle in each dividing cell of the cyst, suggesting a role in the regulation of the cyst cell-division pattern. During divisions, the growing fusome segregates in just one cell of each dividing pair thereby creating an asymmetry in the cyst (Lin and Spradling, 1995). New vesicular material, which fuses with the existing fusome, is generated toward the end of each cell cycle and is first observed in the lumens of nascent ring canals (de Cuevas and Spradling, 1998). Thus, this new fusome material could facilitate the arrest of the cleavage furrow during cytokinesis and subsequent ring-canal establishment. Fusomes disappear at the time cytoplasm transport through the ring canals is initiated within the cyst (Lin *et al*., 1994), but the mechanism of fusome breakdown is unknown.

Genetic studies have supported a requirement for the fusome in cyst formation; mutations in several fly genes that disrupt fusomes lead to defects in cyst formation ranging from complete failure in cystoblast differentiation to cysts with fewer cells that lack a determined oocyte (reviewed in Pepling *et al*., 1999). Cyst development is compromised in females lacking Vasa, Nanos, or Bag-of-marbles (Bam) proteins (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995; Forbes and Lehmann, 1998; Styhler *et al*., 1998). A current model for fusome biogenesis is centered around the regulation of expression of Bam protein that could be influenced by Vasa and Nanos. The function of Bam, a novel protein, is not well understood but recent studies have implicated it in the recruitment of vesicular material to the fusome (Leon and McKearin, 1999). Bam, as well as several other proteins that are components of the membrane-associated actin cytoskeleton, such as ankyrin, α-spectrin, β-spectrin, and Hu-li tai shao (an adducin-like protein), are all found on fusomes. Furthermore, mutations in the *hu-li tai shao* and *α-spectrin* genes eliminate fusomes (Yue and Spradling, 1992; de Cuevas *et al*., 1996). Since filamentous actin is nearly absent from fusomes, these proteins are likely to stabilize the mesh of vesicles in a way that is independent of the microfilamentous cytoskeleton. Fusome biogenesis and correct cyst formation also depend on proteins that interact with the microtubule cytoskeleton. Female germ cells mutant for the cytoplasmic *Dynein heavy chain* gene (*Dhc64C*) or for the *Drosophila* homolog of the lisencephaly disease gene, *lis1*, form cysts with fewer cells and defective or absent fusomes (McGrail and Hays, 1997; Liu *et al*., 1999). The dynein complex and LIS1 are capable of association with spectrin and therefore could be necessary for the attachment of the mitotic spindles to the fusome. Alternatively, these proteins could be involved in the constant transport of vesicles into fusomes.

Although the fusome is required for correct cyst development in *Drosophila*, the presence of a similar germline organelle in organisms other than higher insects has not been confirmed. Thus, it remains unclear whether cyst formation, which seems to be a universal step in animal oogenesis, always depends on a fusome-like structure, or whether this organelle is unique to higher insects where

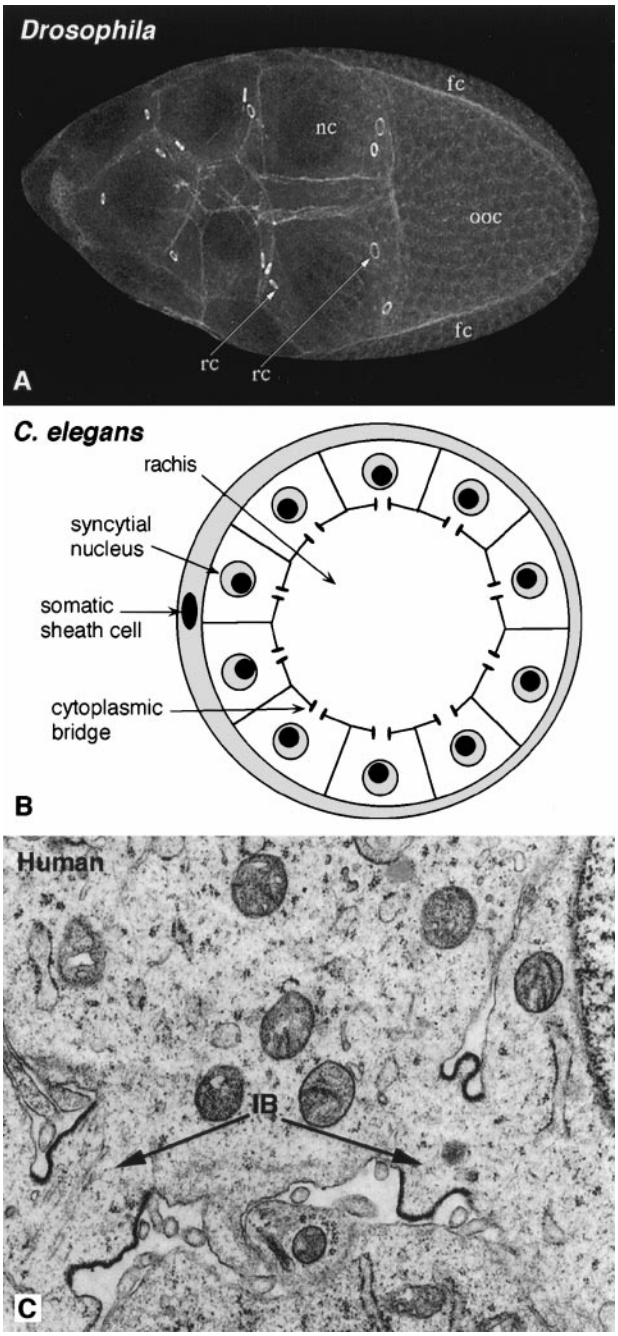

FIG. 3. Syncytial organization of germ cells in *Drosophila*, *C. elegans*, and humans. (A) The germline cyst in *Drosophila melanogaster* consists of one oocyte (ooc) and 15 nurse cells (nc). These 16 cells are interconnected via stable intercellular bridges, also known as ring canals (rc). The cyst is stained for filamentous actin, which lines the subcortical space of the 16 germline cells and the ring canals. The ensemble of 16 germline cells and the monolayer of somatic follicle cells (fc) constitutes the *Drosophila* follicle (also known as an egg chamber). Courtesy of R. Kelso. (B) A cross section of the distal arm of *C. elegans* gonad shows 10 germ-cell nuclei that are circumferentially arranged around a central anucleate core of cytoplasm or rachis. Each individual cell is surrounded by an incomplete plasma membrane and is directly connected to the rachis through a cytoplasmic bridge. Adapted from Gibert *et al.*, 1984. (C) Three human oogonia connected by intercellular bridges (IB) are shown on this electron micrograph. Note the thickened electron-opaque plasma membranes of the intercellular bridges. Reproduced from Gondos, 1987.

divisions are highly patterned and just a single cell in the cyst becomes the oocyte.

Oogonia in *C. elegans*, which are found in the distal arm of the adult gonad, also develop in a syncytium. A cross section of the distal arm shows 8 to 12 nuclei that are arranged as a single layer circumferentially around a central, anucleate core of cytoplasm (also known as the rachis; Hirsh *et al.*, 1976; Gibert *et al.*, 1984; Fig. 3B). Each nucleus is surrounded by cytoplasm and an incomplete plasma membrane that forms a cytoplasmic bridge facing the rachis (Gibert *et al.*, 1984). Consequently, the common core of cytoplasm is continuous with the cytoplasm around the nuclei, thereby allowing free exchange of constituents. The syncytial phase of oogenesis encompasses not only gonial cells but also early oocytes until the pachytene stage of the first meiotic prophase. Even though gonial cells in *C. elegans* share common cytoplasm, they do not form separate clusters as in *Drosophila* or vertebrate organisms (see below). The extent of dissimilarity, though, is unknown since the emergence of the worm germline syncytium and the mechanism of incomplete cellularization are poorly understood events.

The Cyst Phase in Vertebrates

Clusters of gonial cells have been documented in different vertebrate species, including *Xenopus*, zebrafish, chicken, and various mammals (Coggins, 1973; Gondos, 1973b; Ukeshima and Fujimoto, 1991; Selman *et al.*, 1993). Cysts persist while oogonia simultaneously enter meiosis to become oocytes but break down in the pachytene stage of the first meiotic prophase. Therefore, in contrast to *Drosophila*, the cyst phase in these species is confined to early ovarian development. Also, cells in an individual cyst do not differentiate as nurse cells in any of these animals. Instead, each germ cell has the potential to become an oocyte.

In *Xenopus*, oogonia and early oocytes are found as nests of 16 pear-shaped cells that develop synchronously (Coggins, 1973; Gard *et al.*, 1995). The tip of each cell lies at the center of the nest, while the nucleus is located near the periphery. These groups of cells are cysts since individual cells are connected by intercellular bridges that are positioned in the middle of the nest (Coggins, 1973). Oogonia and early meiotic oocytes in the zebrafish are also found as nests of cells that are in the same stage of development. Surprisingly, intercellular bridges have never been observed within the nest (Selman *et al.*, 1993). Thus, zebrafish

oogonia are an example of synchronous development within clusters of cells that may occur without the help of stable intercellular bridges.

Unlike *Xenopus* cysts, chick and mammalian clusters do not have a fixed number of cells. A recent study reported that cells in a mouse oogonial cluster originate from a single cell and often correspond in number to 2^n (Pepling and Spradling, 1998). When stained with a germline-specific antibody, early mouse gonial cells contain round perinuclear structures that are reminiscent of fusome precursors in *Drosophila*. However, these structures neither segregate asymmetrically during cells divisions nor persist throughout the entire period of cluster formation. In addition, examination of the cysts with electron microscopy failed to reveal aggregates of small vesicles. Although the existence of fusome-like structures is questionable, the presence of stable intercellular bridges is well established in the ovaries of birds and a variety of mammals. The intercellular bridges are only 0.5 to 1.0 μm in diameter and their identification has depended on the use of electron microscopy (Zamboni and Gondos, 1968; Gondos, 1973b; Ukeshima and Fujimoto, 1991; Fig. 3C). As in flies, these intercellular bridges have electron-opaque plasma membranes that are thicker than the membranes with which they are continuous; in contrast to flies, they lack an inner proteinaceous layer. Besides temporal distribution of cysts and morphological description of intercellular bridges, little additional information about mammalian cyst development is available, and no components of the intercellular bridges have been identified. Anomalies such as binucleate oocytes, which could result from persistent bridges and subsequent fusion, have been reported (Gondos and Zamboni, 1969). However, mutants with perturbed cyst development have not been characterized. The study of this process is a challenging task since cysts in the mammalian ovary are restricted entirely to fetal and early neonatal development.

Ovarian cyst formation is a conserved and widespread event that probably offers specific advantages to female germ cells. Several hypotheses explaining the necessity for a syncytial phase of development have been discussed in the literature (Gondos, 1973b; Pepling *et al*., 1999). Synchronous development of cells within a cyst creates germ cells at the same stage of maturation that could potentially produce gametes of equal quality. Female germ cells undergo a process of massive cell death while interconnected as well (discussed below). Thus, the cyst arrangement could serve to coordinate both survival and extinction of germ cells. Formation of intercellular bridges within cysts could limit the number of divisions of gonial cells. Studies of certain ovarian tumors, such as disgerminomas, have not revealed intercellular bridges between neoplastic cells even though these cells closely resembled oogonia (Gondos, 1987). Furthermore, several genes in *Drosophila* (*bam* among them), when mutant, result not only in loss of cyst organization but also in unrestricted germline proliferation. Finally, the existence of intercellular connections could provide an opportunity for directed transport toward certain cells of the cyst. This function is clearly the case in *Drosophila* cysts where a constant cytoplasmic stream from the nurse cells supports the developing oocyte. Preferential distribution of organelles and cytoplasm has not been documented for vertebrate oogonia, but more subtle differences in accumulation of certain mRNAs and proteins within a subset of cells in the cyst cannot be excluded.

Incomplete cytokinesis during consecutive cell cycles is a central and conserved event in cyst formation. Yet, the molecular mechanism of the cleavage-furrow arrest in germ cells remains mysterious. Further analyses of genes that are important for cyst formation, combined with studies on the mechanisms of conventional cytokinesis, could shed light on this stage of germline development.

IN OVARIES, FEMALE PGCs AND SOMATIC CELLS ASSEMBLE INTO FOLLICLES

Germ cells interact with somatic cells not only during migration but also after settlement in the gonadal primordium. With the exception of *C. elegans*, in all animal species discussed in this review, gonadal somatic cells envelop female germ cells to create an ensemble called a follicle. Germ cells that are destined to become oocytes have already entered meiosis at the time of follicle formation and within the follicle they reach the diplotene stage of the first meiotic prophase. Here, they halt meiosis for a few days to many years depending on the species. Follicles provide an environment for accumulation of nutrients for future embryogenesis and enormous growth of the oocyte. Formation of mature eggs has not been reported in the absence of follicle structures, suggesting that the support from the gonadal somatic cells is required for the completion of the oocyte differentiation program.

Follicle Formation in Drosophila

Drosophila and other higher insects possess a unique organization of their mature follicles. Gonadal somatic cells envelop the entire cyst of interconnected cells (instead of individual cells) to form a follicle or an egg chamber. Thus, each egg chamber in flies (Fig. 3A) contains a single oocyte, 15 nurse cells, and a monolayer of somatic cells known as follicle cells. In the ovary, egg chambers of consecutive developmental stages are organized in cords or ovarioles (King, 1970; Spradling, 1993). The assembly of an egg chamber occurs near the tip of the ovariole where about 16 follicle cells extend processes to envelop the cyst of 16 germline cells (see Margolis and Spradling, 1995; Goode *et al*., 1996a). Over the course of egg-chamber development, follicle cells continue to divide and form an epithelial

monolayer of about 1200 cells, thereby accommodating the growing germline cyst.

Follicle cells have several essential roles during egg production (reviewed in Spradling, 1993). In the course of differentiation, the follicular monolayer is subdivided into specialized populations (reviewed in Deng and Bownes, 1998) that synthesize a significant amount of the yolk proteins, produce the egg shell, and provide positional cues for embryonic pattern formation.

The origin of follicle cells has been addressed in genetic mosaic analyses. The findings indicate that each ovariole contains on average two somatic stem cells that give rise to all follicle cells (Margolis and Spradling, 1995). These stem cells, however, have not been recognized morphologically. The clonal analysis has also demonstrated that lineage is not important within the follicle cell layer and marked clones often span adjacent egg chambers. Follicle-cell existence does not require the presence of germline cysts, since ovarioles that lack germ cells still have proliferating somatic cells (Margolis and Spradling, 1995).

A variety of intercellular connections exist between neighboring follicle cells in flies. Adherens junctions hold the follicle cells together throughout oogenesis (Mahowald, 1972; Mahowald and Kambysellis, 1980). They are positioned near the apical borders of the follicle cells adjacent to the germline cyst (Peifer *et al*., 1993). Toward the end of oogenesis, septate junctions, the fly equivalent of tight junctions, begin to form just basal to the adherence belt. Follicle cells are interconnected by gap junctions as well. Gap junctions are also present between somatic and germ cells and between the oocyte and nurse cells (Mahowald, 1972; Mahowald and Kambysellis, 1980; Bohrmann and Haas-Assenbaum, 1993). Thus, all cells in the egg chamber are coupled and can exchange small molecules directly. Follicle cells extend microvilli that interdigitate with processes from the nurse cells and the oocyte (Mahowald and Kambysellis, 1980). The number and position of these structures vary during oogenesis, thereby modulating the overall surface of contact dramatically. Finally, stable intercellular bridges have been found not only among germline cells but also between adjacent follicle cells (Woodruff and Tilney, 1998). They are only 0.25 μm in diameter and do not expand in size over time. Also, not all follicle cells are linked by intercellular bridges and the number of interconnected cells in a nest is no greater than eight.

Mutant analyses have revealed a requirement for several *Drosophila* signaling pathways in follicle formation. Hedgehog (Hh) protein, a secreted signaling molecule with roles in cell proliferation and specification of cell identity, stimulates proliferation of follicle cells in the fly ovary (Forbes *et al*., 1996a). This protein is expressed in distinct somatic cells that lie near the tip of each ovariole, two to five cells away from the presumptive follicle stem cells. Ovarioles with a reduced amount of Hh either fail completely in follicle-cell production and do not make egg chambers or produce abnormal egg chambers in which follicle cells envelop more than 16 germline cells. Conversely, ectopic expression of Hh generates a dramatic increase in the number of follicle cells. While Hh and other members of the *hh* pathway function in somatic cells during follicle formation (Forbes *et al*., 1996b), two genes, *egghead* and *brainiac*, are required in the germline cyst for its correct encapsulation (Goode *et al*., 1996a,b). Follicle cells do not properly extend processes toward the cyst when germ cells are mutant for *brainiac* or *egghead* and assembled egg chambers contain multiple oocyte–nurse cell complexes. Although both *brainiac* and *egghead* encode novel proteins, the gene sequences suggest that Egghead could be either secreted or a transmembrane protein, while Brainiac may be a secreted protein. During recruitment of follicle cells to the germline cyst, Brainiac protein may cooperate with the *Drosophila* EGF signaling pathway, which in turn is required both in the germline cyst and in the follicular epithelium for correct egg-chamber architecture (Goode *et al*., 1992, 1996b). Finally, another signaling molecule, the Notch transmembrane receptor, has been implicated in encapsulation of individual germline cysts and specification of subpopulations among follicle cells (Ruohola *et al*., 1991; Xu *et al*., 1992; Goode *et al*., 1996a). Filamentous actin and several actin-associated proteins (profilin, ankyrin, and filamin; Verheyen and Cooley, 1994; de Cuevas *et al*., 1996; Sokol and Cooley, 1999) are enriched in the invaginating follicle cells raising the possibility that follicle-cell migration over the germ-cell cyst may depend on the rearrangement of the microfilamentous cytoskeleton.

The establishment of follicles in the *Drosophila* ovary needs active participation of both the germline cyst and the somatic follicle cells. The involvement of multiple signaling pathways whose specific contribution is not well defined emphasizes the complexity of this process. Genetic screens based on the use of "directed" mosaics, which will allow examination of egg chambers with an entirely mutant follicular monolayer (Duffy *et al*., 1998), could be useful in the identification of more genes necessary for follicle formation in flies.

Follicle Assembly in Vertebrates

In contrast to *Drosophila*, somatic gonadal cells in vertebrates surround a single oocyte to establish a follicle. As in flies, somatic cells extend processes that adhere to germ cells and gradually elongate along them (Gondos, 1969; Ukeshima and Fujimoto, 1991; Fig. 4). Furthermore, follicle cells are intimately associated with the oocyte through numerous interdigitating processes during the growth phase and the entire follicle is coupled with gap junctions (Anderson and Albertini, 1976; Gilula *et al*., 1978; Browne *et al*., 1979; Browne and Werner, 1984; Kessel *et al*., 1985).

In *Xenopus*, newly formed follicles contain a monolayer of squamous follicle cells that are closely apposed to the oolemma (Dumont, 1972; Dumont and Brummett, 1978). Over the course of oocyte growth, cytoplasmic projections from the follicle cells (macrovilli) and the oocyte (mi-

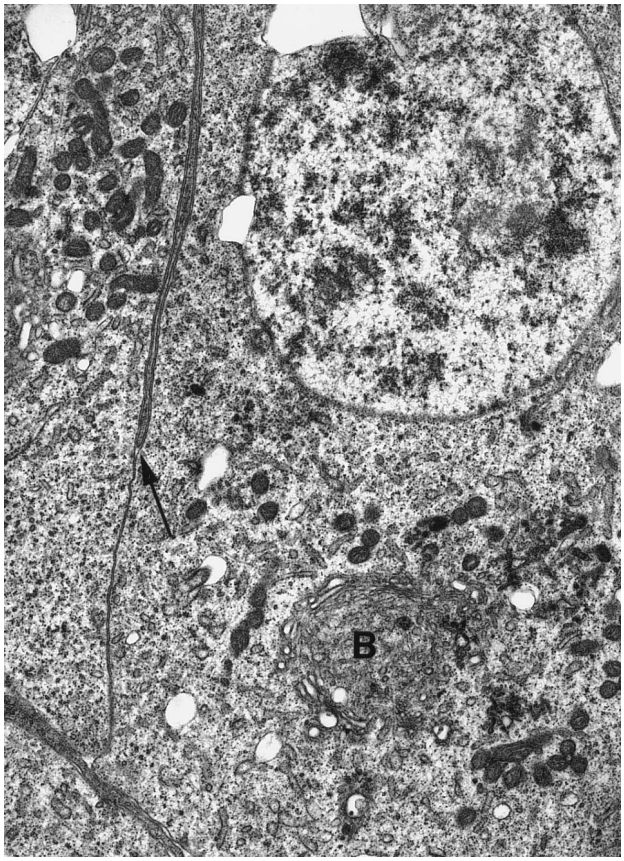

FIG. 4. Follicle assembly in chicks. A germ cell in the left ovary of the 16-day-old chick embryo is in the process of follicle formation. A pseudopodium of a somatic gonadal cell is visible (arrow) that elongates along the surface of the germ cell. A prominent structure in the germ cell is the Balbiani body (B). Reproduced from Ukeshima and Fujimoto, 1991.

crovilli) are readily visible. Simultaneously, follicle cells undergo dynamic changes in morphology. They increase gradually in height to become more cuboidal and then adjacent cells separate from each other to form intercellular spaces. Finally, just prior to oocyte ovulation, follicle cells become flat, most cytoplasmic projections disappear, and contacts between follicle cells and the oocyte are broken.

A single layer of prefollicle cells initially surrounds the entire nest of early meiotic oocytes in the zebrafish ovary (Selman *et al*., 1993). Later, as the cells in the nest cellularize, a sheath of squamous follicle cells surrounds individual oocytes that are in the pachytene stage of the first meiotic prophase. Thereafter, zebrafish follicle cells undergo changes in cell shape similar to those in *Xenopus*. The cells within the zebrafish follicle are in intimate contact during the growth period; long processes extend from the follicle cells toward the oocyte and microvilli originating from the oocyte penetrate deep into the spaces between adjacent follicle cells. While follicle cells withdraw their processes prior to ovulation, microvilli remain on the surface of the ovulated zebrafish egg. The heterotypic adhesion contacts between follicle cells and the oocyte could be mediated by E-cadherin, which together with α- and β-catenins, accumulate subcortically in a punctate pattern along the interacting surface (Cerda *et al*., 1999). Some zebrafish follicle cells seem to have distinct functions; for example, a single specialized cell, called micropylar cell, participates in the formation of the micropyle, the sperm entry point (Hart, 1990; Selman *et al*., 1993). Similar to zebrafish, a micropyle is formed during egg development in *Drosophila* where correct morphogenesis of this structure also requires the participation of a specialized group of follicle cells (Spradling, 1993).

Follicle formation takes an unexpected twist in lizards. Unlike *Xenopus* or zebrafish, the oocytes in the lizard *Anolis carolinensis* are enveloped by multiple layers of follicle cells. Furthermore, a subset of the follicle cells, termed pyriform cells, are connected to the oocyte by intercellular bridges (Neaves, 1971). These connections are an exception from the general rule that intercellular bridges occur only between sister cells, such as germ cells in cysts or *Drosophila* follicle cells. The origin of these bridges is not determined but may be a result of cell-to-cell contact and membrane fusion. Bridges between pyriform cells and the oocyte exist in dormant follicles, but disappear when oocyte growth resumes. The significance of these unusual connections is uncertain; they could facilitate exchange of cytoplasmic materials and maintain the dormant oocytes during periods of suspended metabolic activity.

During fetal or early neonatal development, mammals assemble follicles in a stepwise fashion. As in the zebrafish, mammalian somatic cells first envelop clusters of oocytes to form polyovular follicles (Weakley, 1967). Intercellular bridges are present between oocytes in polyovular mammalian follicles, indicating that somatic cells adhere initially to germ-cell cysts and not to single oocytes. Next, the follicle cells (also known as granulosa cells) send out thin cytoplasmic processes along the germ-cell margin and ultimately encapsulate just one oocyte (Weakley, 1967; Gondos, 1969; Kurilo, 1981). This process marks the assembly of a primordial follicle that consists of a single small oocyte surrounded by a monolayer of a few flattened granulosa cells (Pinkerton *et al*., 1961; reviewed in Hirshfield, 1991). Primordial follicles appear gradually in the juvenile ovary and are often arranged in a cordlike pattern with interfollicular connections that disappear in sexually mature adults (Merchant and Zamboni, 1972). Granulosa cells in primordial follicles change shape from flat to more cuboidal or columnar (Zamboni, 1974; Kurilo, 1981), thus giving rise to primary follicles.

Some primary follicles begin to grow soon after they are formed, while most enter a quiescent state that continues months or years. Concomitant with the onset of oocyte growth, granulosa cells initiate proliferation to enclose the oocyte in several layers. In a period of several days, granulosa cells divide rapidly, while the oocyte retains a constant

size. The resulting multilaminar follicle gradually accumulates extracellular fluid into a large antral cavity and becomes a Graafian follicle. Due to the antral cavity, the oocyte takes up an acentric position and the granulosa cells become divided into two morphologically distinct subpopulations: the inner layer of cumulus granulosa cells that borders the oocyte and the outer layer of mural granulosa cells.

Mosaic analysis in mice has demonstrated that the granulosa cells of individual follicles are oligoclonal in origin and are founded by about five granulosa cells (Telfer *et al*., 1988). A similar number of granulosa cells enveloping a single oocyte has been observed histologically in cross sections of primordial follicles. Granulosa cells in mouse ovarian follicles undergo a dramatic increase in number during oogenesis to reach more than 50,000 in a Graafian follicle. As in *Drosophila* follicles, lineage does not appear to be important within distinct granulosa-cell subpopulations as the cumulus and mural granulosa cells have a common origin (Telfer *et al*., 1988).

Similar to other species discussed here, mammalian granulosa cells are in close contact with each other and the oocyte beginning very early in follicle development. Projections from the granulosa cells invaginate deeply in the ooplasm and end with bulbous terminals that often tightly appose the oocyte nucleus. There, the granulosa-cell terminals establish adherence and gap junctional contacts with the oocyte plasma membrane (Gondos, 1970; Zamboni, 1974). The presence of numerous coated vesicles in the ooplasm has suggested that these close connections could facilitate pinocytic exchange between adjacent cells. The oocyte loses direct physical contact with the associated cumulus cells just before ovulation; nevertheless, a large and expanded mass of cumulus cells embedded in viscous matrix still surrounds the oocyte at ovulation and fertilization. Thus, mammalian oocytes maintain association with somatic gonadal cells at all times.

Hormonal regulation of follicle growth in mammals has been extensively studied (reviewed in Gougeon, 1996). In contrast, knowledge about the molecular mechanisms that govern the early, hormone-independent stages of follicle formation is still fragmentary. Aberrations in packaging, which lead to the formation of polyovular follicles with two or three oocytes, are observed in adult ovaries of a variety of mammals. For example, 14% of all follicles in young dogs and 3% of all follicles in humans are polyovular, suggesting that this phenomenon could be a natural polymorphism (Gondos and Zamboni, 1969; Telfer and Gosden, 1987). Still, the emergence and the biological significance of polyovular follicles are undefined since mutations that result in a penetrant phenotype have not been reported.

Formation or growth of primordial follicles is compromised in several mouse mutants. A failure in the formation of primordial follicles has been reported in mice carrying a null mutation in the germline-specific gene *Figα* (Factor in the germline alpha; Soyal *et al*., 2000). Granulosa cells do not adhere to oocytes lacking Figα protein. As a result, primordial follicles are not assembled, the ovaries are severely shrunken, and the mutant females are sterile. Figα is a basic helix-loop-helix transcription factor, which could be needed for the expression of adhesion molecules on the oocyte or survival factors. Ovarian development arrests at the stage of primordial follicles in mice mutant for the Steel factor (Besmer *et al*., 1993; discussed earlier. Steel factor is expressed in granulosa cells of growing follicles, while its receptor, c-Kit, is present in high levels in oocytes. Therefore, signaling to the oocyte through the c-Kit receptor is required for granulosa-cell proliferation. Similar follicle arrest and subsequent oocyte death have been observed in mice lacking the Growth Differentiation Factor-9 (a TGF-β family member), which is synthesized only in oocytes (Dong *et al*., 1996). In contrast, the cell-cycle regulator cyclin D2 is needed in granulosa cells for their proliferation (Sicinski *et al*., 1996). Finally, female mice lacking connexin 37, a gap-junctional component localized to the oocyte surface, display a profound defect in follicular growth and only small antral follicles are occasionally observed (Simon *et al*., 1997). These mutant follicles lack morphologically distinct gap junctions as well as communication between the oocyte and granulosa cells. The defects seen in *connexin 37* mutants therefore genetically support the long proposed function for gap junctions in follicle growth. Collectively, the mutant studies have demonstrated that both the oocyte and the granulosa cells actively participate in the regulation of granulosa-cell proliferation and oocyte growth.

In summary, ovarian follicles in a number of animal species create a special compartment for germ-cell growth, differentiation, and fertilization. Although the processes of germ-cell encapsulation by somatic cells and the continuous interactions between the oocyte and follicle cells are strikingly similar in diverse organisms, knowledge of genes and signaling pathways involved in follicle assembly is incomplete and the underlying molecular mechanisms remain enigmatic. A systematic study of separate steps in follicle formation and analysis of participating molecules will be an important venue for future research.

PROGRAMMED CELL DEATH IS A WIDESPREAD EVENT IN OVARIAN GERMLINE DEVELOPMENT

Just as cell proliferation is a common step in germline development, so is cell death. As demonstrated in recent years, the demise of germ cells has the features of programmed cell death (PCD) and requires activation of a conserved intracellular program. Death of female germ cells occurs in a predictable temporal and spatial pattern during both invertebrate and vertebrate oogenesis. Female germ cells undergoing PCD (also called apoptosis) manifest a number of characteristic morphological changes such as chromatin aggregation, generation of a highly folded nuclear envelope, cytoplasm condensation, and cell shrink-

age. Despite the recognition of its existence, the biological significance of apoptosis during oogenesis has just begun to emerge.

Requirement for Germ-Cell Apoptosis during Oogenesis in C. elegans and Drosophila

In the nematode *C. elegans*, over half of the female germ cells in the adult gonad are eliminated through apoptosis (Gumienny *et al*., 1999). Cell death affects mainly syncytial oocytes that are about to exit the pachytene stage of meiotic prophase I. The cells that are doomed to die cellularize rapidly, but take away a very limited amount of cytoplasm. Subsequently, they are engulfed by somatic sheath cells that surround the gonad.

Initial understanding of the molecular mechanisms of germ-cell death in *C. elegans* has been helped greatly by research on somatic cell death. Genetic and biochemical studies have outlined a conserved molecular machinery that is responsible for somatic apoptosis in worms, flies, and mammals (reviewed in Metzstein *et al*., 1998; Gross *et al*., 1999; Vaux and Korsmeyer, 1999; Chen and Abrams, 2000). A central event in the execution of PCD in *C. elegans* is the activation of a cysteine protease, the caspase CED-3 (cell death abnormal), from an inactive zymogen that is mediated by CED-4, the worm homolog of mammalian Apaf-1. In living somatic cells, the proapoptotic proteins CED-3 and CED-4 are rendered inactive by the Bcl-2 family member, CED-9. Importantly, the same three genes, *ced-3*, *ced-4*, and *ced-9*, function in death of *C. elegans* oocytes (Gumienny *et al*., 1999). Surprisingly, EGL-1 (egg-laying defective), a protein that inactivates CED-9 to trigger PCD in somatic cells, has no effect on germ-cell death. Therefore, distinct regulatory proteins could control the apoptotic machinery in germline cells. Intriguingly, oocytes of mutants in the ras/MAPK pathway that fail to exit the pachytene stage of meiosis I, also fail to die, suggesting that the meiotic cell-cycle progression influences germ-cell apoptosis (Gumienny *et al*., 1999).

Death of *C. elegans* oocytes is required for the maintenance of germline homeostasis. In *ced-3* or *ced-4* mutants, the number of syncytial germ cells gradually increases (Gumienny *et al*., 1999). However, full-sized oocytes are only occasionally formed. Therefore, excess germ cells that are generated in wild-type animals need to die and "donate" their valuable cytoplasm to the common cytoplasmic core. Only after the dying oocytes pump out almost all of their cytoplasm and cellularize can full-size oocytes be produced from the surviving germ cells.

Germ-cell death occurs at two points during *Drosophila* egg-chamber development. The first is a metabolic checkpoint at the beginning of yolk uptake by the oocyte. The number of germ-cell deaths at this stage is small in young well-fed females, but increases as flies age (Giorgi and Deri, 1976). This event of cell death, which is morphologically reminiscent of apoptosis, affects both the nurse cells and the oocyte. In contrast, the follicle cells are seemingly untouched and engulf portions of germ-cell cytoplasm. Yolk production and uptake are energy-consuming processes and represent a sensitive stage in ovarian development. In aging flies with limited metabolic capacity, the elimination of some previtellogenic egg chambers could serve to curtail egg production. Development of vitellogenic oocytes is also prevented when mating frequency is diminished and mature eggs are retained in the ovary. To accomplish this metabolic checkpoint, female flies could be downregulating the levels of the steroid hormone ecdysone in germline cells, and this reduction could prevent further egg-chamber development (Buszczak *et al*., 1999). Thus, the fruit fly monitors environmental and developmental cues and uses selective cell death prior to vitellogenesis to allow development only of oocytes that will advance successfully through oogenesis.

The second occurrence of germ-cell death is strictly required for the completion of *Drosophila* oogenesis. Once vitellogenesis is accomplished, the nurse cells carry out the fast phase of cytoplasm transport to the oocyte (shown in Figs. 5A–5C). Fast cytoplasm transport is the final event for nurse cells; in 30 min their cytoplasm is depleted and shortly after nurse-cell corpses (Fig. 5C) disappear to leave behind a full-sized oocyte. Thus, within the cyst of 16 interconnected germ cells, only the future egg escapes death.

Final transport is driven by nurse-cell contraction that pushes cytoplasm through the ring canals into the oocyte at an approximate speed of 2 μm/s (Gutzeit and Koppa, 1982). Simultaneously, the ooplasm undergoes circular streaming to distribute the incoming nurse-cell cytoplasm throughout the oocyte. Although failure in final transport of cytoplasm to the oocyte does not prevent completion of oogenesis, the "mature" eggs are very small and never develop.

Nurse-cell contraction is preceded by dramatic morphological changes in the germline cluster. First, nurse cells rearrange their microfilamentous cytoskeleton to form dense arrays of actin bundles around each nucleus. As the cytoplasm of the contracting nurse cells streams, the cages of actin bundles keep the large nurse-cell nuclei in place, away from the ring canals. As a result, nurse-cell cytoplasm freely flows through the ring canals and to the oocyte (Mahajan-Miklos and Cooley, 1994; Guild *et al*., 1997). Next, large masses of condensed nurse-cell chromatin appear throughout the nucleoplasm (Fig. 5E). The nuclear envelopes develop a series of folds (Fig. 5, compare D toE) and gaps (0.5 μm and larger) that allow leakage of nuclear material into the cytoplasm (Okada and Waddington, 1959; Guild *et al*., 1997; Fig. 5, compare A to B). Last, a myosin-driven contraction generates cytoplasm flow through the ring canals and leads to shrinkage of the nurse-cell cluster (Wheatley *et al*., 1995; Edwards and Kiehart, 1996). Taken together, the morphological alterations in nurse cells before and during fast cytoplasm transport have suggested that nurse-cell regression is an example of programmed cell death.

Molecular and genetic analyses have further supported

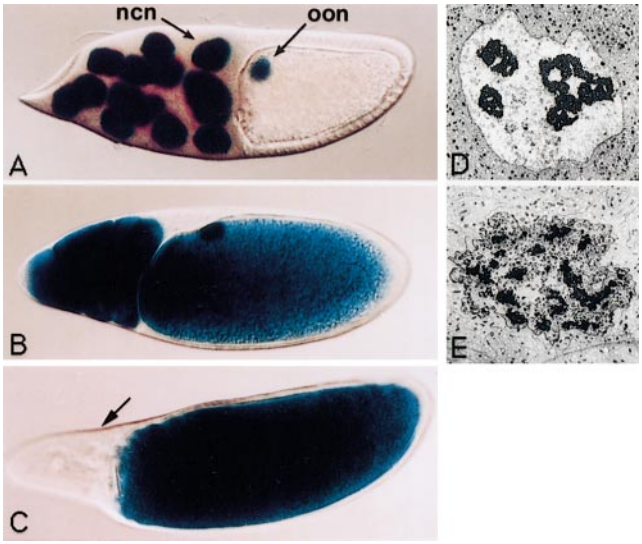

FIG. 5. Germ-cell apoptosis in *Drosophila*. (A–C) Regressing nurse cells during consecutive stages of final cytoplasm transport in *Drosophila*. β-galactosidase expression from a germline-specific transcriptional reporter visualizes cytoplasm transport. β-galactosidase activity is initially contained within the nurse-cell nuclei (ncn) and the oocyte nucleus (oon) due to a nuclear localization signal in the fusion protein (A). Later, the nurse-cell nuclear envelopes become permeable and β-galactosidase activity is found in the cytoplasm as well (B). Finally, nurse cells contract and cytoplasm rapidly flows into the oocyte resulting in doubling of the oocyte volume and regression of the nurse-cell cluster (C). The arrow in panel C points toward nurse-cell corpses. Adapted from Cooley *et al*., 1992. (D) This electron micrograph shows a nurse-cell nucleus prior to the onset of final cytoplasm transport. The nucleus contains dense masses that resemble nucleoli. (E) This electron micrograph demonstrates the morphology of a nurse-cell nucleus at the onset of final cytoplasm transport. The nuclear surface becomes highly indented and chromatin aggregates are visible throughout the nucleoplasm. (D and E) Reproduced from Guild *et al*., 1997.

the apoptotic nature of nurse-cell regression. A change in cytochrome c distribution is one of the earliest reported molecular indicators of apoptosis in cultured cells (Liu *et al*., 1996). In nurse cells, an overt alteration in the conformation of cytochrome c, evidenced by the exposure of an otherwise hidden epitope, occurs prior to nurse-cell contraction (Varkey *et al*., 1999). Fragmentation of DNA, which is characteristic of apoptosis, is detected within the nurse-cell nuclei (Cavaliere *et al*., 1998; Foley and Cooley, 1998). Finally, nurse cells that lack the *Drosophila* caspase protein-1 (DCP-1), a CED-3 homolog, are defective in fast cytoplasm transport due to a failed contraction. Furthermore, these mutant nurse cells are also unable to reorganize their actin cytoskeleton and to permeabilize their nuclear envelopes (McCall and Steller, 1998). Collectively, these findings have provided genetic evidence that an apoptotic signal triggers all morphological changes in nurse cells and is responsible for the generation of contraction. The transcripts of two other caspases, named Dredd and DRONC, accumulate in egg chambers at a time coincident with nurse-cell death, suggesting that these cysteine proteases may also play a role in nurse-cell regression (Chen *et al*., 1998; Dorstyn *et al*., 1999). Surprisingly, three proapoptotic proteins, Reaper, Head Involution Defective, and Grim, which promote caspase activation in somatic cells, are not required for nurse-cell apoptosis (Foley and Cooley, 1998). Thus, as in *C. elegans* germ cells, a separate set of regulatory proteins is likely to activate the basic cell-death machinery in *Drosophila* nurse cells.

Once active, caspases like DCP-1 cleave a specific set of effector proteins. Although the substrates of DCP-1 in nurse cells remain elusive, downstream proteins that are involved either in actin reorganization or in contraction have been investigated both genetically and biochemically. These studies have demonstrated that the morphological changes that follow DCP-1 activation can be genetically separated (see Matova *et al*., 1999). Several actin-binding proteins participate either in actin polymerization (Profilin) or in subsequent bundling of actin filaments (Quail and Fascin). When nurse cells are mutant for any of these actin-binding proteins, formation of actin cages around the nurse-cell nuclei fails; however, permeabilization of the nurse-cell nuclear envelopes and subsequent contraction are unaffected. Hence, it has been concluded that the reorganization of the microfilamentous cytoskeleton is one of the genetic pathways that is triggered by caspase activation. Conversely, when nurse cells have reduced levels of the regulatory light chain of nonmuscle myosin II (encoded by *spaghetti squash*), contraction fails but rearrangement of the actin cytoskeleton shows no obvious defects (Wheatley *et al*., 1995; Edwards and Kiehart, 1996). This finding therefore outlined a second genetic pathway downstream of DCP-1. The active DCP-1 could directly cleave nuclear lamins (see McCall and Steller, 1998), thereby promoting destabilization of the nurse-cell nuclear envelopes. Consequently, a release of calcium from the nuclear envelopes could lead to capacitative calcium entry, which in turn could initiate a myosin-based cortical contraction (Jordan and Karess, 1997; Matova *et al*., 1999).

Besides *Drosophila*, apoptosis of germ cells is likely to occur in other insect species where oocytes are fed by nurse cells. For example, in hymenopteran insects (e.g., wasps), nurse cells also "empty" their entire cytoplasmic content into the oocyte toward the end of oogenesis. In addition to characteristic alterations in nuclear morphology, rings of intricately arranged microtubules (Bilinski and Jaglarz, 1999) or filamentous actin (Gutzeit and Huebner, 1986) are formed around the nuclei. Thus, the process of germ-cell death in different insect species involves reorganization of either the microfilamentous or the microtubule cytoskeleton, but the specificity of this choice is unknown.

Apoptosis of germ cells in worms and flies is a part of normal germline development and is required for fertility and maintenance of homeostasis. Paradoxically, death of

germ cells in these organisms is a creative rather than a destructive process. Even though many germ cells are cleared, the outcome is the production of full-sized oocytes, which acquire the precious cytoplasm from cells that die. Although participation of components of the core cell-death machinery is established, the molecules that specify death and control caspase activation await discovery.

Germ-Cell Death in Vertebrate Ovaries

Death of germ cells during ovarian development has been reported in several vertebrate classes as well. In *Xenopus*, oocytes that have initiated yolk accumulation are particularly vulnerable to cell death. At this stage, dying oocytes that appear mottled with swirls of yolk and pigment have been detected in the ovary (Dumont, 1972). Oocyte death also can be induced by starvation. Thus, as in *Drosophila*, yolk formation or uptake is a sensitive point in frog oogenesis, and mature eggs are produced only under optimal conditions.

In chick embryos, normal ovarian development is dramatically different between the right and left sides. While the left ovary consists of an inner medulla and an outer cortex, the right ovary fails in cortex formation, stops further development, and gradually regresses. Germ cells in the right and left ovaries have distinct fates as well (Ukeshima and Fujimoto, 1991). Although proliferation and cyst formation are alike in both sides, germ cells in the right ovary do not proceed through meiosis and are rarely enclosed by somatic cells. Dying germ cells are observed in the medullae of both ovaries but the frequency of cell death is much higher in the right. Notably, apoptotic germ cells are not seen in the cortex of the functional left ovary. Electron-microscopic examinations have shown that the dying germ cells, which are mainly observed as a germ-cell mass, display the characteristic features of apoptotic death and are engulfed by adjacent epithelial cells or macrophages (Ukeshima, 1996). Intriguingly, chick germ cells undergo apoptosis while they are still organized in cysts.

It has long been known that death of germ cells is a prominent feature of mammalian ovarian development. In fetal and newborn ovaries, the extensive process of direct germ-cell death has been referred to as attrition. Later in life, germ cells are mostly lost after death of the supporting follicle cells, an event that is known as follicular atresia. Recent ultrastructural and molecular studies have classified both attrition and atresia as apoptotic phenomena (Coucouvanis *et al*., 1993; De Pol *et al*., 1997; Kaipia and Hsueh, 1997; Morita and Tilly, 1999).

In rat prenatal ovaries, germ cells proliferate to reach a peak number of 64,000 normal oogonia at 17.5 dpc (Beaumont and Mandl, 1961). At birth, however, the number of healthy oocytes falls to about 39,000, and by 2 days postpartum (pp) the count declines to 19,000 oocytes. Developing human ovaries show similar dynamics of germ-cell death (Baker, 1963). The total population of germ cells reaches a peak of 6,800,000 at 5 months postconception. By the time of birth, the number of germ cells drops precipitously to 2,000,000, of which only 50% are healthy cells. The process of germ-cell apoptosis continues in postnatal life so that only 300,000 oocytes survive at 7 years pp and fewer than 1000 are present in the years just prior to menopause (Baker, 1963; Wise *et al*., 1996). Since a healthy woman will ovulate approximately 400 eggs during adult life, the ultimate fate for the vast majority of germ cells (99.994%) is death. Thus, ovulation and fertilization are rare events that rescue only a very few eggs from death and provide the possibility of a new life.

Human and rat germ cells undergo apoptosis at three different points of fetal and neonatal ovarian development. The first "wave" of death that affects mitotic oogonia (Beaumont and Mandl, 1961; Baker, 1963) is controversial since in some investigations the number of dying oogonia is reported as negligible (Kurilo, 1981). The other two waves of germ-cell apoptosis are well established to occur in oocytes in the pachytene and the diplotene stages of meiotic prophase I (Beaumont and Mandl, 1961; Baker, 1963; Kurilo, 1981). Therefore, germ cells commencing meiosis are particularly susceptible to cell death. Characteristic morphological changes seen in germ cells include chromosome fusion, contraction of chromatin material, wrinkling of the nuclear envelope, and cell shrinkage. While pachytene oocytes are not enclosed in follicles, diplotene oocytes participate in a very active process of follicle formation and only 14% are not found within primordial follicles (Kurilo, 1981). Thus, two important ovarian processes, meiosis and folliculogenesis, intersect with extensive germ-cell death. Whether these processes contribute to the selection of subsets of oocytes that will survive or die remains unclear.

In human fetal ovaries, germ cells in early stages of apoptosis are frequently connected by intercellular bridges (Gondos, 1973a). Conjoined cells, which are found in pairs or groups, often show similar regressive changes. Intercellular bridges are rarely observed during advanced stages of germ-cell death; when present, these bridges are distorted and partially disrupted. Curiously, even late in apoptosis when intercellular bridges are no longer seen, adjacent dying cells have a similar appearance (Fig. 6). Thus, the signal for germ-cell death seems to be generated and propagated while germ cells are still syncytial. As apoptosis progresses, though, the affected cells cellularize. Therefore, the initiation of the synchronous wave of ovarian germ-cell death could be greatly facilitated by the existing intercellular bridges. However, survival of germ cells might be dependent on timely separation of individual oocytes from the syncytial group; only then might these individual cells create the pool of surviving oocytes.

Targeted gene disruptions and overexpression studies of several members of the core death machinery have further supported the apoptotic nature of germ-cell attrition and follicular atresia. Even though female mice that are mutant for *bcl-2*, the prototypical cell-death protecting gene, are fertile and have normal litter numbers, their ovarian architecture is abnormal (Ratts *et al*., 1995). The number of

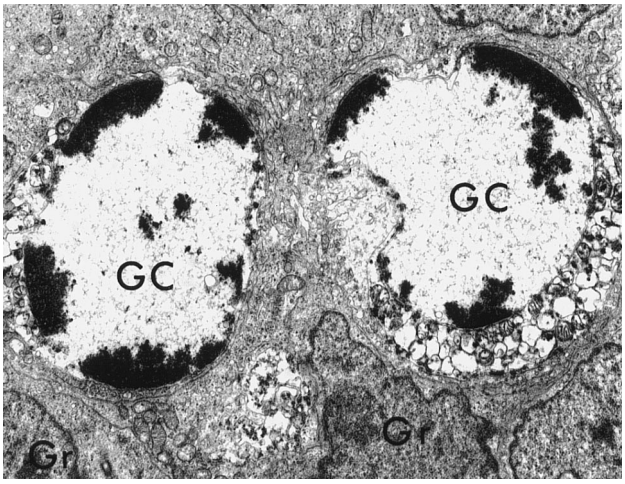

FIG. 6. Apoptosis of human germ cells. The electron micrograph shows two adjacent human germ cells (GC) in advanced apoptosis that display similar nuclear and cytoplasmic regressive changes. Granulosa cells (Gr) surround the dying germ cells. Reproduced from Gondos, 1973a.

healthy primordial follicles is significantly reduced in these mutant animals. Closer examination has revealed numerous aberrant primordial follicles that contain a single granulosa cell layer without an oocyte or with a dying oocyte. Therefore, *bcl-2* is required for the endowment of a normal complement of germ cells in the mammalian ovary. The role of *bcl-2* in follicular atresia has been investigated using targeted overexpression in granulosa cells (Hsu *et al*., 1996). These transgenic animals have a decreased rate of follicle atresia, enhanced folliculogenesis, and a larger litter size. However, improved survival of granulosa cells could cause germ-cell tumorigenesis since some aging mice develop benign cystic ovarian teratomas. Therefore, apoptosis in the mammalian ovary, as in worms and flies, could be necessary for the maintenance of germline homeostasis.

The role of two proapoptotic proteins, Bax and Caspase-2, in ovarian apoptosis has also been studied in mutant animals. Young mice deficient for Bax, a protein that antagonizes Bcl-2 function, are fertile. Furthermore, these mutant females possess threefold more primordial follicles than their wild-type sisters and the surplus of follicles is maintained to advanced age (Perez *et al*., 1999). Mutant females do not have a greater initial endowment of oocytes, suggesting a normal rate of attrition. The incidence of follicular atresia, however, is significantly lower in Bax-deficient females. Although aged females fail to ovulate and become pregnant due to age-related decline in hypothalamic-hypophyseal function, eggs retrieved through superovulation are competent for *in vitro* fertilization and early embryogenesis. Therefore, ovarian lifespan can be prolonged without obvious germline defects by selective disruption of Bax function. Unlike Bax-deficient animals, mice lacking the cysteine protease Caspase-2 have attenuated germ-cell attrition that results in a higher initial number of primordial follicles (Bergeron *et al*., 1998). Nevertheless, the mutant females do not have apparent fertility problems. Caspase-2-deficient oocytes are also resistant to drug-induced apoptosis, demonstrating that this protein mediates both normal and pathological apoptosis of germ cells.

The balance between germ-cell survival and apoptosis during fetal development has been investigated recently in mice that are doubly mutant for *bcl-x* (a cell-death protecting gene) and *bax* (Rucker *et al*., 2000). Females that carry a hypomorphic mutation in *bcl-x* show a severely reduced population of primordial and primary follicles and greatly impaired fertility. The *bcl-x* mutant phenotype can be corrected when both copies of the *bax* gene are deleted. As a result, these double-mutant females have a normal rate of germ-cell survival, reproductive life span, and litter size.

Taken together, the mutant analyses have confirmed that both attrition and follicle atresia are events of programmed cell death. The experimental findings, however, are still insufficient to explain the necessity for massive cell death in mammalian ovaries. Follicular atresia could be beneficial for aging individuals, but this hardly is the only reason for the demise of thousands ovarian cells starting as early as fetal development. The effects of too much or too little cell death may be enhanced when more than one protein with synergistic functions in apoptosis are removed from the ovary. These experiments combined with quantitative analysis will address the issue of redundancy during ovarian apoptosis.

In conclusion, extensive programmed cell death takes place during normal ovarian development of both invertebrate and vertebrate organisms. Direct germ-cell death in ovaries is initiated while cells are still interconnected. The mechanism of execution is likely to be conserved across species and involves the same molecules that function in somatic cell death. In contrast, molecules that activate the core apoptotic machinery in germ cells are likely to be different from those known in somatic cells. An important objective for future studies is the elucidation of the signals that trigger apoptosis in germ cells. A limited supply of extracellular survival signals or selection for best-fed cells are some of the mechanisms that could initiate germ-cell death; still, the specific molecules that carry out these functions are largely undefined.

GENERAL CONCLUSIONS

The process of oogenesis has been the subject of debates ever since humans have seen animals hatching from eggs. Not until the Scientific Revolution in the 17th century was it accepted that all animals develop from fertilized eggs. At that time the English physician William Harvey (1578–1657) focused attention on oogenesis by postulating that "all that is alive comes from the egg" or in his words "*ex ovo omnia* " (see Pinto-Correia, 1997). Today, this concept is not questioned; instead, accumulated knowledge on

oogenesis in diverse species has fostered the idea not only that all animals develop from eggs, but also that they make eggs using common strategies. The extent of the similarities in oogenesis among different animal species is uncertain and many questions are still open for investigation. The complete knowledge of the genome of *C. elegans*, *Drosophila*, and human in conjunction with other technological advances provide remarkable opportunities to study comparative aspects of oogenesis. The identification of homologous proteins in different species is becoming a routine task. Providing evidence for functional conservation of these molecules remains a challenging, yet obligatory step for further progress. The egg has not lost our attention; after all, each of us came from one.

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